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CYTOLOGICAL AND LEUKEMIO-PATHOLOGICAL PROBLEMS CONCERNING THE YOSHIDA SARCOMA (A MONOCYTIC TUMOR OF THE RAT)

(With Plates I and II)

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INTRODUCTION

Yoshida reported a new transplantable tumor of the rat under the title of "the new strain of transplantable ascites sarcoma" (Yoshida, Muta. Sasaki, 1944). According to his own abstract: "This tumor shows the morphological picture corresponding to reticulosarcoma, and, cytologically, the cell is several times as large as the leucocytes; its indented nucleus leans to one side, and a neutral-red rosette exists near the indentation of the nucleus; the oxidase reaction is weakly positive, and as to the size and the form of the nucleus it varies to a great extent, but it is interesting to compare this cell with the lymphocyte or monocyte." After the examination of the smears, Amano, our leader and cooperator, insisted that this tumor should be classified equally with the monocytic tumor, i. e., green monocytoma (so-called chloroma) or not-green monocytoma (in further anaplastic state in which the cell ceases to produce the green pigment), based on the similarity of the character of this tumor cell to that of the monocyt. (Amano: Fundamental Problems of Hematology, 1948). As to the cytological character of this tumor cell, Yoshida once referred it to the histiocyte, while afterwards he thought that it belongs probably to the monocyte, which opinion was generally accepted (Yoshida, 1947, Special-report in the Jap. Path. Soc.).

Hamazaki, however, from a new view-point, asserted that this tumor should be regarded as the coelothelioma of the abdominal cavity (1948, Discussion at the Jap. Cancer Association; 1949, and his report "a cytological study on Yoshida-sarcuma," Acta Hemat. Jap.). If this tumor is the coelothelioma of the abdominal cavity, how can we understand the following properties of this tumor cell, namely to be free in the abdominal cavity, and consequently, the possibility to proliferate freely in the fluid of the body, without any direct supporting tissue? According to our opinion, the monocyte performs its function and cell-division not only in the blood but also in the tissues, that is, the monocyte is the special cell which is the cell of the blood and also that of the tissue, (Amano, 1943, Special

Lecture at the 6th meeting of the Jap. Hemat. Soc.), and from this fact we can understand its mode of proliferation like chloroma. Consequently, the tumor cell being monocytogenous, the Yoshida sarcoma should show the mode of proliferation similar to leukemia, but few studies are made as to this point. Yoshida himself stated that he could produce the metastatic focus only in the heart and the kidney by the transplantation of the tumor cell into the blood stream, without resulting any leukemic change (1948.). Recently, Kimura et al. made an experiment to transplant the Yoshida sarcoma to the blood stream with the result that they could find the tumor cells in the blood to ca. 50 %. And this should be regarded as the blood picture that is comparable with the chloroma. But the difference between the result of these two experiments is too big!

We have ascertained precisely the cytological properties of the Yoshida sarcoma, and, subsequently through the intravenous transplantation of these tumor cells, we got very interesting leukemiopathological findings in the blood and tissue pictures.

Cytology

We transplantated the Yoshida sarcoma into the abdominal cavity of the rats (ca, 80—90 gr), and thereafter, examined daily its cytological picture by smear preparations or supravital staining with neutral-red-janusgreen, etc. According to his classification Yoshida divided the cellular nature in the course of transplantation to the abdominal cavity into three phases. In the first phase, we also could find small cells whose nucleus has the apparent tendency to lobulate, and, in the second and the third phases, there are seen large typical tumor cells with comparatively round nucleus. The main difference between the two latter phases is as follows: the azur granules are gathering in the second phase, while those granules are distributed in the third phase. In short, according to Yoshida's opinion, the tumor cells proliferate taking the typical shape of the monocyte soon after the transplantation, then these cells metamorphose gradually into the typical tumor cells. This process is thought to be repeated every time after the transplantation. From our findings, though the tumor cells are somewhat shrunk and small at first, and the nucleus indicates apparent unevenness, there exist in the tumor cells special, large nucleoli, and a more or less fine and brownish-tinged neutral-red rosette, that are common to the tumor cells in all phases (Fig. 1). So we can not agree with tumor cell cycle in the peritoneal cavity as Yoshida understands it, for in that case he must have enumerated normal monocyte reaction in peritoneal cavity (Hirata, 1946), as tumor cells, to some extent. The above-mentioned conditions are proved even from the fact that these monocytes in the first phase indicate 70 to 80% positive in the peroxidase reaction (by

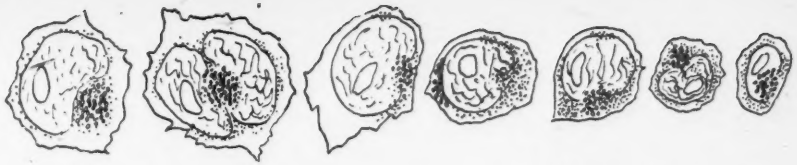

Fig. 1: Supravital staining (small dots—janus-green, vacuoles—neutral-red rosette.)

Sato-Sekiya's method). The tumor cells, examined in the second phase when their proliferation is prosperous, are 15 to 25 % positive in the peroxidase reaction (Fig. 2). Naturally the peroxidase reaction is narrower is its extent of appearance

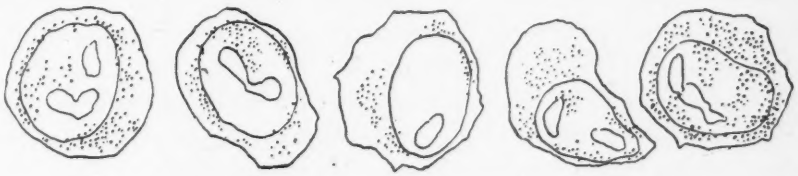

Fig. 2: Peroxidase reaction of the tumor cells

than the oxidase reaction, and, moreover, as the recent research told us the Yoshida-sarcoma cells were less positive in the oxidase reaction than when they were examined during the study in early time. So we expected that the peroxidase reaction might be negative in the present study, but at any rate, we gained an apparent positivity though only slight. This finding is the important one to those who examine the various changes of the patients of leukemia. Furthermore, we injected China-ink into the abdominal cavity of rats at the end of the second phase and punctured out the fluid 24 hours later. With this punctured fluid we observed the tumor cells through supravital staining, and we found that the carbon particles were absorbed at the surface of the neutral-red vacuoles. Therefore, the phagocytosis for the ink-particles is obviously positive (Fig. 3). When observed in the dark field, we found barr-shaped, short, thin projections from the protoplasm, that are similar to those of normal monocytes. During our observation under the warmth-holding equipment (Fig. 4), we observed the changes taking place in the protoplasm as shown below. The character of the tumor

Fig. 3: Phagocytosis of carbon-particles

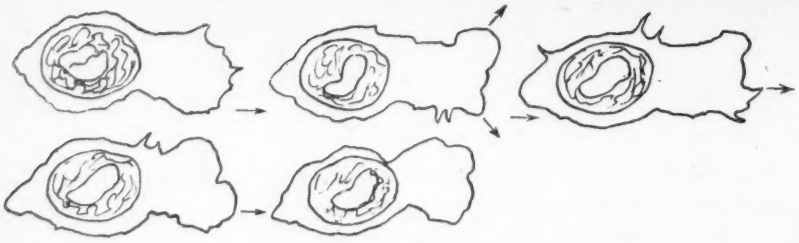

Fig. 4: Movement of tumor cells

cells resembles generally those of the monocytes, especially those of the monocytes in the phase of leukemia, and they look not at all like those of the other sort. So we can not regard this tumor as a coelothelioma of the abdominal cavity (Hamazaki) any more. Our various findings of the tumor cells such as positive peroxidase reaction, phagocytosis of carbon-particles, formation of ciliary processes and movement of protoplasm, etc., are not coinciding with Yoshida's description. Instead of this, it was more exactly ascertained that the mother cells of this tumor are monocytes, and there may be no further objection against this fact.

We have seen that the properties of this tumor cell coincide with those of the monocyte in all points, but there coexist among them some strongly anaplastic monocytes which are sometimes seen in the case of human monocytic leukemia, and which transitionally lacks a neutral-red rosette, the phagocytosis being more slight or negative and the peroxidase reaction being negative. Between these anaplastic monocytes and the tumor cells with the above-mentioned properties, we can see an obvious transition from a certain view-point concerning the character of the protoplasm and, especially of the huge nucleus. Naegeli (1931) and his followers, who insist that the monocytes are the cells which differentiated from the myeloblasts, deny the independent presence of monocytic leukemia, and in some cases, even when the picture is obviously monocytic they still insist stubbornly that those leukemic cells would soon metamorphose into myeloblasts at the terminal period. But from the present fact that the monocytes proliferate in a state that appears like the sarcoma, when judged from the exterior, while they do not make anaplasia to the myeloblasts, we shall be able to point out the contradiction in Naegeli's theory ; the mother cell of the monocyte is the monoblast, and not the myeloblast ; and the monocytic system is an independent blood system apart from the myelogenous-granulocytic system. With the appreciation of the cytological character of this tumor, our theory receives another proof.

Leukemia-Like Changes Produced Through Intravenous Transplantation of Tumor Cells

Allowing the tumor cells as monocytes, it may be possible to set up a monocyte-leukemic change in animals using these tumor cells. But, if the sarcomatous properties of the tumor cells are stronger, we must expect some modification in this leukemoid reaction.

At first, we divided twenty rats (ca. 70—120 gr) into two groups, and examined the blood picture respectively the day before the transplantation. The next day, we injected from the saphenal vein 0.2 cc of ascitic fluid containing tumor cells (diluted six times with Ringer-solution: 30000 cells/cmm). This procedure is done in two times on account of the calculation of the blood cells. Formerly we decided to kill the animals every three days after the transplantation, but most of the animals in which we could inject tumor cells regularly died 7—9 days after the transplantation, the others survived until to the 12 th to 15 th day, while one rat lived until the 20 th day.

a) Pathological findings:

We killed only four tumor-transplantated rats after three, four, seven and fifteen days, the other rats having died. There were some which were killed in the agonizing state before death. As it was during the very cold season, our organ smears were comparatively successful.

It was in the case after the sixth day that an enlargement of the liver and the spleen were recognized at the dissection. On the contrary in those animals that survived comparatively long over 12 days, the enlargement of those organs were not apparent, but the distention of the thymus and lymphatic glands was very obvious. So it seemed to us that the metastasis to the lymphatic apparatus took place without any connection with the enlargement of the liver and the spleen. Of course, the distention of the lymphatic glands was seen in the whole body, especially the axillary, para-aortic and mesenterial glands. In two cases, we ascertained the distention of the renal capsule with tumor tissues.

b) Histological findings: (See Table 1a, 1b.)

In the case of the rat which was killed after three days, no distended organ was found macroscopically, but we observed histologically that the tumor cells were building bridgeheads in all organs, among which the obvious were seen in lymphatic glands, lung (around the small veins), kidney (renal pelvis, submucosa, localization around the glomeruli), etc. But in the liver we found a few shrunken tumor cells in the Glissonian capsule. In the spleen the tumor cells exist as a localized embolus, but in the bone marrow it was difficult to find out cytologically the neoplastic focus with accuracy, for the neutrophilic young cells in

bone marrow are generally large and hardly distinguishable from the tumor cells. Instead of this we could agree that the normal picture of the bone marrow suffered no damage whatever, judging from the distribution of sinus and megalocytes. As to the two rats of the second group which were killed after four and seven days respectively, we observed similar findings.

As to the cases after six—eight days, we found a notable cell infiltration in the liver, the spleen and the bone marrow, and these cell-infiltrated organs were apt to indicate the histological nature of leukemia when compared with the other organs; that is to say, there was a notable tumor cell-infiltration in the Glissonian capsule, and in the intralobular sinusoid. Moreover, we could also observe the active phagocytosis of the swollen stellated cells against the intruded tumor cells. In the spleen, we saw a diffuse distribution of the tumor cells in the red pulp; the follicles were atrophic. This picture of cell-infiltration is far stronger than in the acute monocytic leukemia of the human autopsy. In the bone marrow, the formation of tumor noduli became gradually apparent, (this can be judged from the disappearance of the peculiar structure), though their margins were not sharp but diffusely melting into the surrounding marrow. Besides, the kidney, the lymphatic glands, etc., which formerly called our attention revealed no further change; in the lung, the embolism of the tumor cells increased in the small pulmonary veins and the picture of edema and hemorrhage was seen around it. Many of these tumor cells of the embolus decreased the capacity of the staining of the nuclei and turned to degenerate. It may be possible that such cells were found in the peripheral blood to some extent (in smear preparation of the blood).

After seven days, the liver, the spleen and the bone marrow indicated the complete leukemic picture: in the sinusoid of the liver we found sound tumor cells, in the splenic pulp the tumor cells increased so much as to press the perifollicular zone, the bone marrow lost its own peculiar structure and the tumor cells proliferated often in a state like that of reticulum cells. But the process seemed not so malignant as to indicate a remarkable absorption of the bony beams.

Now, in this period, the peculiar structure of the lymphatic glands began gradually to be lost and to be substituted with tumor cells. In the kidney, the astonishing fact is that in every glomerulus we found the capillaries full of tumor cells, a picture which is only really seen in the large-cell monocytic leukemia. But as we found, in many cases, no tumor cell in the capillaries of the renal tubules, we should think that this phenomenon was not due to the increase of the tumor cells in the blood, but to the embolism of them. We found some emboli of the tumor cells in every part of the pulmonary arterioles, some of

Table 1a Experiment 1

Table 1a Experiment 1

Organs / Number	Heart	Lung	Liver	Spleen	Kidney	Lymphatic Glands	Bone marrow	Other organs
1 3 d	n. p.	Tc embol. in artt. & perivenous infilt.	G. cap. (Tc ±) ca. 5.5 g.	Pulp (Tc +) Artt. (Tc ±) ca. 0.5 g.	Periglom (Tc +) Renal pelvis (Tc +), periartt. (Tc +)	Med. pulp. (Tc +)	N. p.	
5 6 d	In auricular blood (Tc +)	Similar to the above	9.2 g. G. cap. 5 (Tc ++) Sinusoid (Tc +)	1.6 g. Pulp (Tc ++)	Peripyel. (Tc +)	(Tc +)	(Tc +)	
9 7 d	N. p.	Embol. in artt. (Tc +) & focal pneumonia.	12.3 g. G. cap. (Tc +++) Sinusoid (Tc +++)	2.2 g. Pulp (Tc +++)	Glom. capil. (Tc ++)	(Tc ++)	(Tc +)	
4 7 d	In auricular blood (Tc +)	Emcol. in artt. (Tc ++) & lymphatic (Tc +)	11.7 g. Similar to the above.	2.2 g. Similar to the above.	Similar to the above.	(Tc ++), structure confused.	(Tc +)	
3 8 d	Embol in small arteria coronalis (Tc ++)	Embol. in artt. (Tc ++), venous walls destroyed.	12.1 g. Similar to the above.	1.4 g. Pulp (Tc ++) Foll. atrophic & erytropoiesis (+)	Interst. of pelvis (Tc ++), glom. capil. (Tc ++).	Slight cortical infilt., Tc occupied 3/4.	Pulp (Tc ++)	
7 8 d	In capil. (Tc ±),	Similar to the above.	10.9 g. Similar to the above	1.8 g. Pulp (Tc +++), hemorrhagic. Foll. atrophic.	In similar place respectively (Tc +).	Chiefly in cortical sinusoid (Tc +).	Similar to the above.	
8 9 d	Similar to the above.	Embol. in artt., perivascular infilt (Tc ++).	10.8 g. Similar to the above, sinusoid destroyed and hemorrhagic.	2.1 g. Similar to the above, erythropoiesis (+).	In similar place resp. (Tc +~++).	Similar to the above (Tc +~+++).	1/2 occupied.	
2 15 d	N. p.	N. p.	5.1 g. n. p.	0.6 g. (Tc ?)	Knotlike infilt. in pelvis, calcium deposit in tubules.	Similar to the above (Tc +).	(Tc ±)	
6 15 d	In capil. (Tc +)	Localized tumor infilt. in subpleural & peribronchial regions, embol. in artt. (Tc ±).	6.3 g. G. cap. (Tc +), Sinusoid (Tc +), localized.	0.6 g. Phagocytes in foll., pulp (Tc +), & necrosis of arteries.	Glom. capil. (Tc +).	Cortical nodes atrophic foamy cells 3/4.	1/5 occupied by Tc.	Complicated unknown disease.
10 20 d	Tc in capill. (±).	Similar to the above.	7.3 g. Similar to the above.	0.9 g. Foll. atrophic, pulp (Tc ±), knotlike infilt, around foll. Extensive degeneration of central arteries.	Similar to the above (Tc +~++).	Pulp to cap. (Tc +++).	(Tc +), own structure relatively kept.	Similar to the above.

Table 1b Experiment 2

Organ / Number	Heart	Lung	Liver	Spleen	Kidney	Lymphatic glands	Bone marrow	Other organs
11 4 d	In capil. (Tc —)	Perivenous (Tc ⊥)	5.8 g.	0.7 g.	N. p.	N. p.	Almost normal.	
12 7 d	Similar to the above.	Peribronchial & perivenous (Tc ⊥).	5.4 g. Only in G. cap. (Tc ⊥).	0.7 g. Pulp (Tc ⊥), sinus extended.	Interst. of the pelvis (Tc ÷).	Cortical sinus (Tc ⊥∼÷), 1/7 occupied.	(Tc ⊥)	
17 8 d	In capil. (Tc ⊥)	Embol. in artt. (Tc ⫩), peribronchial (Tc⫩).	16.4 g. G. cap. & sinusoid (Tc ⫩∼⫼).	1.9 g. Foll. atrophic, sinus (Tc ⫩), erythropoiesis (⊥)	Embol. in glom. capil. (Tc ⫩).	Tc occupied 5/6 of the gland.	Spotty distribution of Tc.	
13 8 d	Similar to the above.	Similar to the above, Tc degenerated. (⫩)	19.6 g. G. cap. (Tc ⫼) Sinusoid (Tc ⫩) hemorrhagic.	2.35 g. Foll. atrophic, pulp (Tc ⫩), erythropoiesis (÷)	Tc infilt around the ureter, (⫩), in glom. capil. (÷).	Cortical nodes destroyed by Tc	Completely occupied by Tc.	
16 9 d	Similar to the above.	Somewhat stronger than the above.	7.95 g. G. cap. (Tc ⫼) Sinusoid (Tc ⫩∼⫼) Typical leukemic liver.	1.3 g. Foll. (Tc ⫼), pulp (Tc ⫼), erythropoiesis (÷).	Embol. in glom. capil. (÷), hemorrhagic.	Tc 4/5.	Tc 7/10.	
19 9 d	Similar to the above.	Similar to the above.	10.6 g. G. cap. (Tc ⫼), sinusoid (Tc ⫩), spotted necrosis of liver cells.	1.95 g. Foll. atrophic (Tc around them) Sinus destroyed & hemorrhagic (Tc ⫼) & erythropoiesis (⫩).	Pelvis & cap. (⫩), embol. in glom. capil. (⫩) some Tc & calcium deposit in tubules.	Foamy cells 3/4, Tc 1/4 of infilt. in cap. (⫼) in one gland, in another Tc 4/5.	Almost Tc.	
20 12 d	Among m. fibers (Tc ⫩).	Big knotlike tumors in the subpleural & peribronchial regions, sharply margined.	6.9 g. Localized & sharp-margined focus in G. cap. (Tc ÷) sinusoid (Tc ⊥), erythropoiesis (÷).	0.8 g. Foll. n.p., hemorrhage in perifollicular zone, (Tc ÷), Pulp (Tc ⫩∼⫼), erythropoiesis (⫩).	Interst. of pelvis & its cap. (Tc ⫼), embol. in glom. capil. (Tc ÷∼⫩).	Almost Tc.	Almost Tc & Hemorrhagic.	Mediastinal tumor including thymus & lymph glands.
14 13 d	In capil. (Tc ⊥)	Embol. in artt. (Tc ⊥), peribronchial (Tc ⊥), but not so large.	5.7 g. Sinusoid (Tc ⊥∼÷), knotlike sharp-margined tumor around the G, cap. (÷).	1.05 g. Similar to the above.	Broad infilt. to the area that implys ureter & renal pelvis, & glom. capil. (Tc ÷∼⫩)	Almost Tc & infilt. to the surroundings.	Almost Tc.	Similar to the above and skin (Tc ⫩).

d—days after embol—embolism artt—arterioles cap.—capsule infilt.—infiltration n.p.—nothing particular g.—gram
Tc—tumor cells G. cap.—Glissonian capsule capil.—capillaries glom.—glomeruli foll.—follicles m.—muscle med.—medullary
interst.—interstitium peripycl.—peripycelitic

which going into degeneration.

As to those rats that died after twelve, thirteen, fifteen, twenty days, etc, they did not show any notable enlargement of the liver and the spleen, but histologically we could find tumor cells in every organ of the body, and the findings concorded more or less with that of the rat killed after three days, but, as described above, also in these cases the metastatic enlargement of the lymphatic glands was seen to a high extent and the proliferation of the tumor cells in the surrounding of the cortex of the thymus, the renal capsule and the general lymphatic apparatus was very intense.

Observing more precisely, we found that the metastasis in the liver was almost limited to the Glissonian capsule. There were some tumor cells which were very characteristic as to their shrunken and strongly stained nuclei, their extended protoplasm, and the tumor cells were sometimes so inactive that they gave us the impression as if they were driven to bay. And in the spleen we found no such infiltration as to cover the whole structure of the pulp. But in the bone marrow, the tumor cells proliferated day after day and almost occupied the whole marrow cavity; in some part the bony beams were pressed to become atrophic. Sometimes we saw that the actively dividing cells were connected with each other with protoplasm-binding projection. From this point of view, we can state that the tumor cells in the bone marrow proliferate lineally like those in the lymphatic apparatus. As to the findings of the lung and the kidney they were fairly similar to those of the rats killed after three days.

Summarizing these findings, in the cases after twelve days, we can say that the tumor cells cease to proliferate further, but stay defensive in the metastatic position. On the other side we may say that an anti-neoplastic property is aroused in the blood. Furthermore, we may suppose that, in spite of the increased anti-neoplastic property in the blood, the tumors in the lymphatic glands or in the bone marrow proliferate indifferently, and so consequently the animals die. It is often said that the metastatic tumors in the lymphatic glands are stolid to the X-ray, but here are two examples which indicate that the blood differs very much from the lymph, though they are equally humoral.

Then, what is tbe factor which inhibits the growth of the tumor? If it is an antibody against the collapsing tumor in the blood (apparent in the lung, the liver, as well as in the peripheral blood), this fact may be explainable. Takeda

Table 2a Experiment 1

		WBC	N	E	B	L	M	P	TC	Erybl	Notes
1	0 d	19500	12.7	2.0	0.0	77.0	8.3	0.0	0.0	0	K 3 d af.
100 g	2 d	13850	9.7	6.7	0.0	65.7	16.3	0.0	1.7	0	
5	0 d	15600	9.7	4.7	0.0	67.4	18.1	0.0	0.0	100	D 6 d af.
100 g											
9	0 d	20600	56.2	1.0	0.0	26.5	16.3	0.0	0.0	0	D 7 d af.
110 g	3 d	24350	20.0	1.0	0.3	59.7	17.0	0.0	2.0	50	
4	0 d	13250	5.3	1.7	0.0	79.0	14.0	0.0	0.0	0	D 7 d af.
110 g	4 d	17900	18.5	2.7	0.0	57.1	18.2	0.0	3.7	200	
3	0 d	20000	10.7	0.3	0.0	81.0	8.0	0.0	0.0	0	D 8 d af.
115 g											
7	0 d	12100	18.7	6.0	0.0	55.7	19.7	0.0	0.0	0	D 9 d af.
100 g	4 d	21950	13.7	7.0	0.0	53.0	22.3	0.0	4.0	0	
8	0 d	18850	31.7	4.7	0.0	47.0	16.7	0.0	0.0	0	D 9 d af.
95 g											
	0 d	24500	19.0	2.7	0.0	69.3	9.0	0.0	0.0	0	
2	5 d	20800	24.3	1.7	0.0	51.7	19.3	0.0	2.7	0	K 15 d af.
95 g	10 d	21100	25.0	4.3	0.0	54.7	14.3	0.0	1.7	0	
	15 d	18750	18.7	6.7	0.0	58.3	16.0	0.0	0.3	0	
6	0 d	17350	16.4	2.1	0.0	66.5	15.8	0.0	0.0	0	D 15 d af.
115 g	4 d	16100	21.7	3.7	0.0	57.7	15.0	0.0	2.0	0	
10	8 d	18400	29.7	1.3	0.0	50.7	15.3	0.0	3.0	0	
120 g	0 d	18950	17.0	0.0	0.0	62.5	20.5	0.0	0.0	0	D 20 d af.
	8 d	14400	16.0	0.3	0.0	64.7	18.0	0.0	1.0	0	

Injected volume.....0,2 cc (6 times diluted ascitic fluid with physiologic NaCl solution, intravenous injection.). Cell count.....29900/cm. But we diluted the injection-solution to a higher extent in case No. 10.

Table 2b Experiment 2

		WBC	N	E	B	L	M	P	TC	Erybl	Notes
11	0 d	9100	34.3	2.7	0.0	44.3	18.7	0.0	0.0	0	K 4 d af.
90 g	4 d	10250	22.3	1.7	0.0	53.7	22.3	0.0	0.0	0	
12	0 d	11750	22.3	3.3	0.0	50.3	24.0	0.0	0.0	0	K 7 d af.
100 g	4 d	12500	17.3	1.3	0.0	66.7	14.7	0.0	0.0	0	
	7 d	14350	27.3	2.3	0.0	52.3	17.3	0.0	0.7	0	
17	0 d	10500	22.7	0.7	0.0	60.3	16.3	0.0	0.0	50	D 8 d af.
100 g	5 d	23000	16.0	1.0	0.0	69.3	13.0	0.0	0.0	100	
13	0 d	9300	45.3	1.7	0.0	39.7	13.3	0.0	0.0	0	D 8 d af.
110 g	4 d	23900	43.0	0.3	0.0	39.7	16.7	0.0	0.3	0	
	7 d	17450	36.0	1.0	0.0	43.0	20.0	0.0	0.0	50	
16	0 d	11600	33.5	0.0	0.0	52.0	14.5	0.0	0.0	250	D 9 d af.
70 g	5 d	12300	31.7	2.7	0.0	52.7	13.0	0.0	0.0	0	
	8 d	27750	38.3	1.7	0.0	48.0	11.0	0.0	1.0	350	
19	0 d	94500	18.3	0.7	0.0	65.0	16.0	0.0	0.0	0	D 9 d af.
85 g	6 d	14000	28.0	2.7	0.0	60.3	9.0	0.0	0.0	50	
	8 d	28050	32.0	3.0	0 0	35.0	22.5	0.5	6.5	9550	
	0 d	11000	32.3	0.0	0.0	56.3	11.3	0.0	0.0	0	
20	6 d	10600	19.3	3.3	0.0	59.7	17.7	0.0	0.0	0	D 12 d af.
105 g	8 d	18000	31.3	1.7	0.0	47.0	19.0	0.0	1.0	200	
	10 d	14100	27.5	0.5	0.0	49.5	20.5	0.0	2.0	6800	
	11 d	40950	9.0	0.5	0.0	68.5	14.0	0.0	8.0	690	
	0 d	12350	9.7	0.7	0.0	72.3	17.3	0.0	0.0	0	
14	5 d	15050	17.0	1.0	0.0	68.7	13.0	0.0	0.3	50	D 13 d af.
95 g	8 d	21900	33.7	2.0	0.0	51.7	12.7	0.0	0.0	0	
	10 d	24600	18.0	0.0	0.0	56.0	25.0	0.0	1.0	1950	
	11 d	25350	34.0	2.0	0.0	52.0	12.0	0.0	0.0	750	

The same solution as in Exp. 1. No. 11...No. 14 (31250/cmm)
No. 16...No. 20 (29350/cmm)

d.....days, af.....after, g.....gram, K.....killed, D.....died, WBC.....white blood cell, N.....neutrophil, E.....eosinophil, B.....basophil, L.....lymphocyte, M..... monocyte, P.....plasmacyte, TC.....tumor cell, Erybl.....erythroblast/cmm

(1949) was examining this relation, using the rats of the pure strain, but our rats are of mixed strain, so we could not examine it more profoundly. Concerning the tumor which is transplanted into the blood, there appeared a probable hematogenous metastasis of the tumor into the major omentum and the mesenterium in the medium stage. We examined the major omentum or the mesenterium by the Giemsa-staining after they were extended on slides and dried. But the picture of these preparations differed somewhat from those of the blood, rather they were similar to those of the metastatic tumor in lymphatic glands.

c) Hematological findings:

The results of the blood examination of the rats tested intravitally are shown in Table 2.

The percentage of the tumor cells which appeared in the peripheral blood reached almost ca. 10% but it was not so high as Kimura et al. (1948) have mentioned before. There were many rats that showed nearly 20%-presence of monocytes both before and after the experiment. We paied much attention in order not to take this monocyte for the tumor cell. There were many rats whose blood contained still 20% of monocytes though the tumor cells increased. Why did in the case of Kimura et al., the monocytes decrease very much while the tumor cells increased (about 50%)? This is the point that we could not understand from our experiments. Even though we took their classification and enrolled the whole monocytes into the tumor cell count, yet we could obtain only less than 30%. But, as the blood of the rat abounds in lymphocytes, there is some possibility to miscount, but in this case we are rather sure not to have done such mistake.

In short, the percentage of the tumor cells in blood was expected to be fairly high from the histological picture, but really it was near 10%, i. e., the examined blood indicated mere subleukemic picture. This may be due to the fact that the tumor cells increased in the size and in the sarcomatous character, and appeared slightly in the peripheral blood, as they increased the histiotropic affinity more than the hemotropic affinity.

Further, in the case after the tenth day from the transplantation we often recognized erythroblastosis (See the table), but no young myelogenous cells appeared during that time. We could observe the orthochromatic and basophilic erythroblasts along with above-mentioned fact, when we compared the tissue slides with the tissue smears. In these cases we could easily observe the distribution of the erythroblasts in the perifollicular sinus of the spleen and in the sinusoid of the liver, the tumor metastasis in the spleen and the liver were not so prominent in those cases. This distribution tells itself that the erythropoiesis went through the metastasis. It was very interesting from the hematological

point of view, that we could prove this fact in materials such as tissue-preparations, organ and blood smears side by side. In our cases, the bone marrow was csmpletely occupied by the tumor cells and even the boney beams were going to become atrophic and absorbed. But the intruded tumor cells had shrunken and strongly stained nuclei as well as transparent protoplasm. On account of those results we may say that they were cytologically in a similar defensive state as in the case of the liver metastasis of the tumor.

Conclusion

According to our tissue-slide preparation, supravital observation and histochemical examination, the Yoshida sarcoma provides the character of the monocytes in every point (the formation of the neutral-red rosette, as well as that of protoplasmic projection, and the movement of protoplasm). Contrary to previous reports, according to our experimental technics, the tumor cells show phagocytosis against the carbon particles; furthermore the peroxidase reaction is positive. Therefore, it was decided that the Yoshida sarcoma belonged to the monocytic tumor. The existence of such monocytic tumor really denies the standpoint of the Nagelian scholars who insist that the monocyte is merely a differentiated cell from the myeloblast, and, at the same time, we may say that it supports the independence of the monocytic system, and of course we can not agree with Hamazaki's opinion that this tumor is the coelothelioma of the abdominal cavity.

When we transplant the cells of this tumor into the blood stream, we can obtain the pathological picture which accords with general monocytic leukemia about a week after, along with the subleukemic blood picture. Though we expected higher leukemic properties judging from the histological findings, in reality, the tumor cells in the blood were less than 10%, which told us that the hemotropic affinity of these big cells decreased, while the sarcomatous properties increased.

We can classify two processes of tumor distribution, considering attitudes of the tumor cells in hematopoietic organs. The tumor cells which continue the proliferation from beginning to end step by step (from three to twenty days) are those in the lymphatic apparatus and the bone marrow, while those in the liver and the spleen, abruptly proliferate prosperously in the medium stage. The proliferation in the latter organs causes the leukemic picture, killing the animals in seven or nine days. In the cases survived twelve days after the transplantation, the enlargement of the liver and the spleen is slight, the further growth of the tumor cells ceases and they remain conservative in the spleen as well as in the liver. There are morphological signs that certain humoral inhibitory factors

are produced, though the tumor cells proliferate indifferently in the lymphatic apparatus. In the comparatively long-survived rats (more than nine days) the appearance of erythroblasts in the peripheral blood reached a high degree. In such cases we could ascertain extra-medullary proliferation foci, which were perhaps metastatic, in the organ sections and smear preparations. In these cases, the bone marrow was completely occupied by the tumor cells.

As to these findings, we can point out many similarities to those of the human leukemia, but, in general, the sarcomatous properties of this tumor are fairly strong; it varies between two categories, i. e., that of leukemia and that of leukosarcomatosis.

In addition to this experiment here reported, we are still examining the further properties of the Yoshida sarcoma by transplanting tumor cells directly into the bone marrow.

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Legend for Plate I:

Fig. 1: Carbon particle phagocytosis of tumor cells (supravital staining, neutral-red, 5 days after the intraperitoneal transplantation).
Fig. 2: Seen in the dark field. The neutral-red vacuoles are very remarkable.
Fig. 3: Beginning of tumor infiltration in the liver (intralobular infiltration after 6 days). See the active state of Kupffer cells.
Fig. 4: Complete picture of leukemic liver (after 7 days).

Fig. 5: Conservative infiltration in the port-biliary capsules of the liver (after 12 days).
Fig. 6: Liver, localized infiltration (after 13 days).

Legend for Plate II:

Fig. 7: Kidney, tumor cells in and around glomeruli (after 7 day).
Fig. 8: Lung, tumor embolus in arterioles (after 6 days).
Fig. 9: Lung, localized metastasis of tumor cells in terminal stage (after 12 days).
Fig. 10: Bone marrow, complete tumor metastasis (after 9 days).
Fig. 11: Tumor cells in peripheral blood (degenerated cells, May-Grunwald-Giemsa staining, 8 days after the intravenous transplantation).
Fig. 12: Erythroblastic reaction in the peripheral blood (May-Gruenwald-Giemsa staining, 8 days after the intravenous transplantation).

要　旨

吉田肉腫に関する細胞学的並に白血病病理学的諸問題に就いて

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吉田肉腫の細胞学的性格に就いては，單球を母細胞と見る見解が一般に承認されているが，最近濱崎氏は此腫瘍を腹腔上皮腫と見做す見解を発表している。若し此腫瘍が腹腔上皮腫であれば，此腫瘍が腹腔内に遊離して発育し易い性状に対して理解が困難となる。我々の見解によれば單球は血液の細胞であると共に組織の細胞たるの二面性をもつ特有の細胞であり，このことが綠色腫の如き増殖様式を採るものと解せられる。從て單球に属するならば吉田肉腫は白血病類似の増殖様式をなさねばならないのであるが，今日迄の研究ではその点は充分吟味されていず，吉田氏は血液内移植により白血性を呈せしめ得ることなく僅に心と腎に轉移竈を作り得たというに反し，木村氏等は50％に近い腫瘍細胞の血液内流出を認めている。しかし両者の成績の相違は余りに甚しく，我々は是に改めて吉田肉腫の單球としての細胞学的性格を精細に検索し，併せて血液内移植による探究を行うこととした。

吉田氏の見解では腹腔内移植の初めは單球型細胞として増殖し，その細胞が日を経て一定の腫瘍細胞に移りゆくとするのであるが，我々の腹腔内移植の所見よりすれば，成程移植初期の腫瘍細胞は萎縮して小型であり，核にも凹凸が著しい傾向はあるが，しかし腫瘍細胞には特有の大型の核仁が存在し，中性赤ロゼツテも小粒で，帶褐色の色調を呈している点で第Ⅱ期のものと本質的に異るところはない。そして吉田氏が第Ⅰ期に増加すると称するものは腹腔常備の乳斑單球が刺戟を受けたためによるものと考えられる。此事は過酸化酵素反應で此種の單球が70～80％陽性であることからも裏付けられる。腫瘍細胞は第Ⅱ期の旺盛な増殖相にある時期に検した所では，15～25％程度が本反應陽性である。此所見は白血病患者の諸反應を試みている者の目にとつては決して見逃し難い所見である。其上墨粒の腹腔内注射後24時間の超生体染色所見では，墨粒は中性赤空胞面に吸着し，明かに墨粒貪喰能は陽性である。又暗視野所見で胞体に短い細い，いが栗状の突起が出ているのも一般單球と一致した性質である。尙保溫装置の下では胞体の運動をも併せて観察することが出來た。此等の腫瘍細胞の性格は一般に單球，殊に白血病時の單球に酷似してをり，他の如何なる種類の細胞とも似ないものであつて，本腫瘍が單球を母体とするものであることは最早疑問の余地のない所である。而して此様な單球系の腫瘍が実在することは，Naegeli 学派の單球を骨髄芽球の一分化細胞と観る立場を否定し，單

球系の独立性を支持するものということが出來る。

血液内移植においては7～9日頃に斃れるものが最も多く，屠殺したのは4例であつた。

最も早く屠殺した3日後例では肉眼的に腫瘤性臓器はなかつたが組織学的に既に全身に腫瘍細胞が橋頭堡を作つてをり，殊に淋巴腺，肺（小靜脉周囲），腎（腎盂粘膜下，糸毬体局在性），脾，肝（グ鞘），等において判然と認められた。

6日以後になると肝脾腫が起り，組織学的にも今迄轉移の著明でなかつた肝，脾，骨髄に著明な浸潤が認められ，他臓器を引き離して白血病様の像に赴いている。即ち肝臓のグ鞘には相当著明な腫瘍浸潤があり，靜脉竇にも次第に充満してゆく。脾では髄索に腫瘍細胞が瀰蔓性に認められ，濾胞は萎縮性となり，遂には該細胞が髄索内に充満する。かかる像は人の急性單球白血病の場合よりも強力な浸潤振である。肺では靜脉に腫瘍の栓塞が多くなり，これを囲んで浮腫，出血も見られる。

腎ではどの糸毬体にも毛細管内に腫瘍細胞が栓子となつて充満してをり，かかる所見は我々は稀に大型の細胞の單球白血病に見るのみである。淋巴腺所見は以前のままで骨髄では次第に腫瘍の結節を作りつつ浸潤し，遂には骨髄は固有の像を失い腫瘍細胞は細網細胞様型態で増殖する。

12日以後では，いずれも肝脾腫は著明でないが，組織像では矢張り全身臓器に腫瘍細胞を認めた。仔細に観ると肝の轉移は殆どグ鞘に限られ，腫瘍細胞も核が濃縮性で胞体が廣く，活氣のない細胞群からなり，又脾臓にも髄索を掩う程の浸潤なく，肺も同様の限局性の腫瘍轉移竈を認めた。一方淋巴装置では淋巴腺の高度腫大，胸腺周囲並に皮質における腫瘍の増殖を認め，骨髄ではその殆どを腫瘍細胞により占められ，その中の一部は漸く骨梁の圧迫萎縮をさへ起さんとしている。

此所見は要約すれば，肝，脾その他では一旦増殖した腫瘍細胞が直線的な発育を止めて，轉移の位置で守勢を保つているに過ぎないことを思はせる。強いていへば血液中に抗腫瘍性が生じたともいうべき所見である。しかも淋巴腺，骨髄での直線的増殖は，淋巴腺轉移腫瘍がX線に鈍感であるといわれる点をも考慮すれば，等しく体液的環境でも血液と淋巴とでは甚だしく相違があるということが推測され得る。而してこの様な腫瘍発育の抑制因子は血液中で崩壊し続ける腫瘍に対する抗体が生ずるものとすれば，此事実は若干説明が可能であろう。

日を逐つて調べた血液像では腫瘍細胞の血中出現率はまず10%が最高で亞白血病性といえる所見で，木村氏のいう如く高率ではなかつた。組織像から案じて，もつと発現率が高くともよいと思われるが，これは腫瘍細胞が大であり，且肉腫的性格を増して，血液親和性よりも，組織親和性を増し，末梢血中の動員が少かつた爲であると考えられる。事実血中の腫瘍細胞も変

性せるものが多かつた。尙移植10日以後を経た例では，屢々高度の赤芽球症を認めた。しかし幼若骨髄性細胞は認められなかつた。これ等の例は，脾，肝の腫瘍轉位は軽微であるが，骨髄は屢々腫瘍細胞の爲に完全に占拠され，骨梁すら萎縮，吸收に傾きつつある。しかも腫瘍細胞は濃縮性核の，胞体の透明な細胞よりなり，その点で肝の守勢にある轉移と同一の細胞学的態度にある。

吉田肉腫の此等の所見は人の白血病と比べて多くの点で類似性のあるものであるが，概していえば，肉腫性強く，白血病と白血性肉腫との二範疇の間を彷徨する性質のものである。

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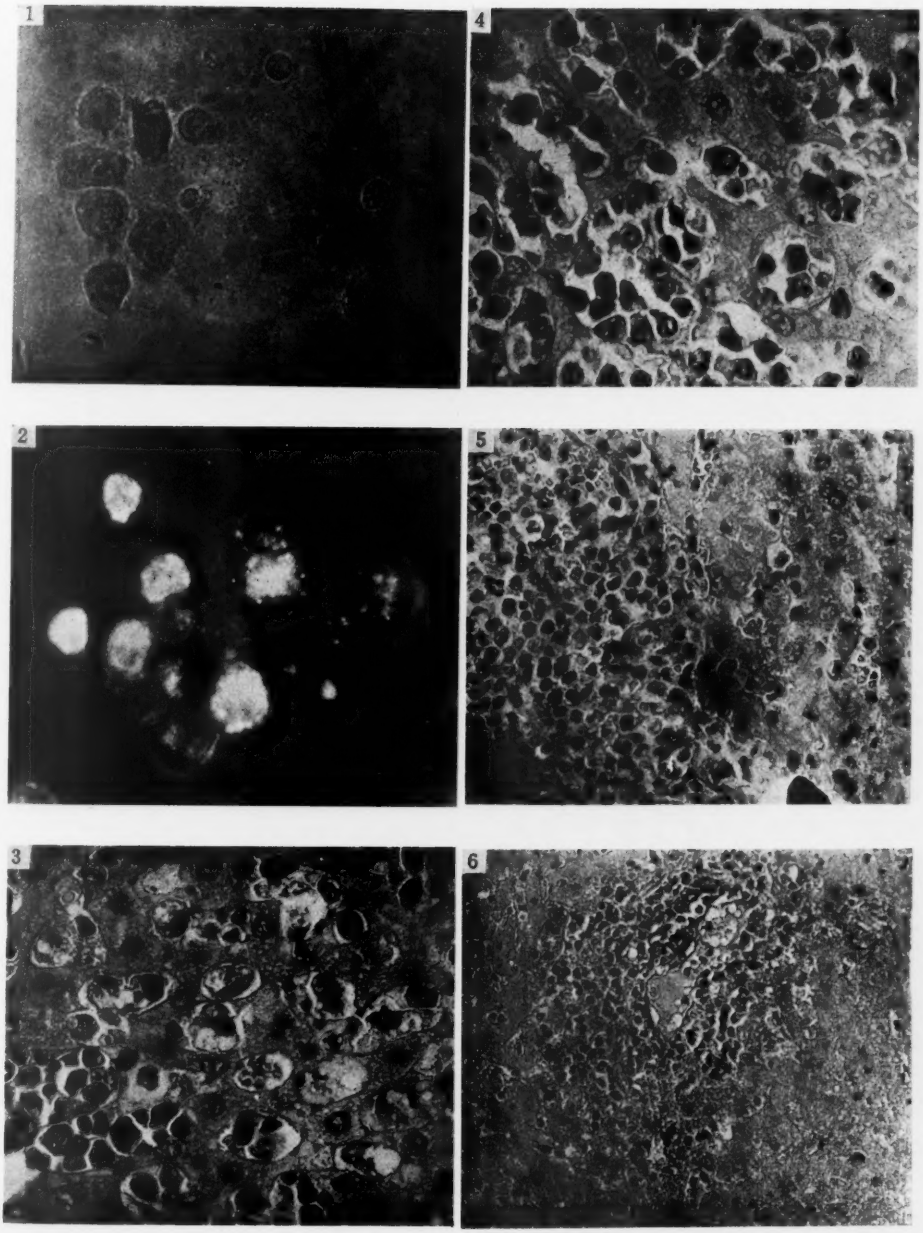
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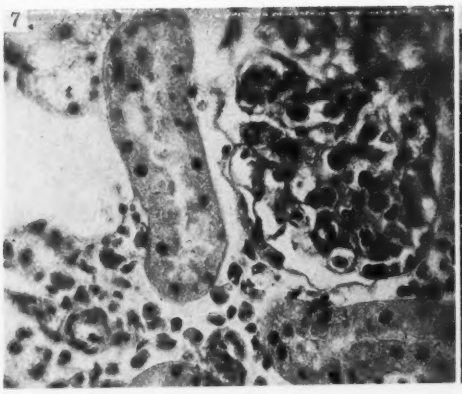
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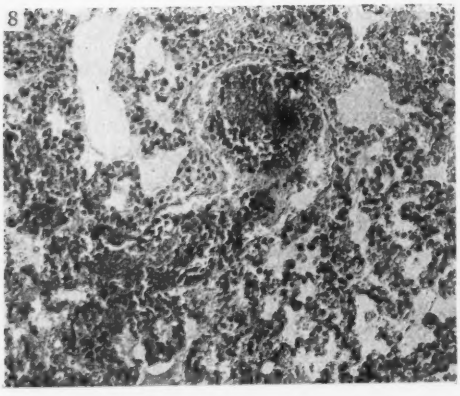
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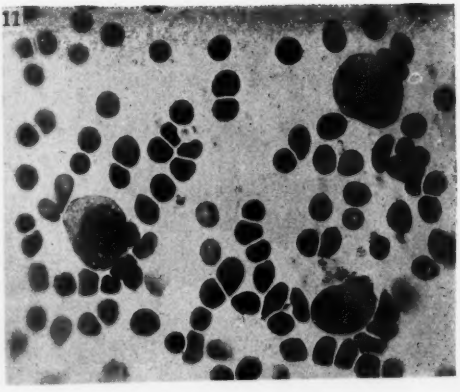
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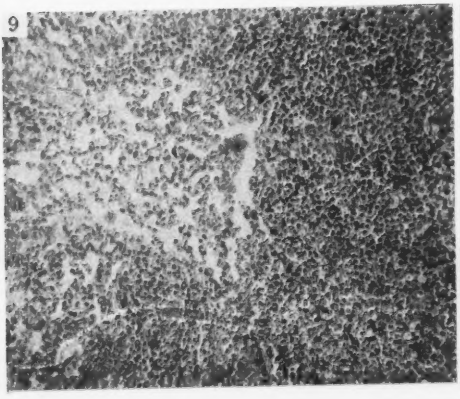
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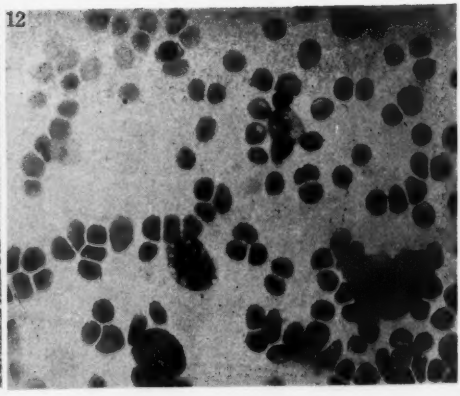
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[GANN, Vol. 42; March 1951]

STUDIES ON IMMUNOLOGICAL PHENOMENA IN MALIGNANT TUMORS

(Experimental Studies with Yoshida Sarcoma)

(**With Plates III and IV**)

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INTRODUCTION

Yoshida Sarcoma (1) is a tumor with a peculiar way of propagation. It is a fluid tumor, as we call it, and proliferates in such a condition as having tumor cells suspended in the ascites. This enables us to perform such experiments as have hitherto been found difficult technically with ordinary nodular tumors. The tumor transplantation experiment with a single cell which Ishibashi (2) tried with success in our laboratory was a practical illustration of the fact.

The research in the field of the immunological phenomena of malignant tumors has not much been pursued. The following are references to this found in the medical literature. Tanabe (3) reported that he had tried experiments using Fujinawa Sarcoma and found the re-transplantation checked distinctly. Ishiwara (4) conducted similar experiments with three different groups of tumors—Kato Sarcoma (group 1), Jensen Sarcoma (group 2) and Flexner Cancer (group 3). In group 1 there was hardly any immunity conferred, while in groups 2 and 3 the growth of retransplanted tumors was distinctly stunted, in most cases giving no sign of growth at all. The reporters of cases where tumors disappeared or lessened after successive injections of tumor vaccine were Blumenthal (5) and his co-operators, Ono (6) and Nakamura (7). Carl Lewin (8) observed complete extinction of tumors after injecting several times into tumor animals 1 cc. of serum from spontaneously cured rats. Akao (9), who attempted similar experiments, acknowledged a great effect of such sera upon tumors. In short, it has been known that immunological phenomena may be observed, to some degree, among tumor cells. However, were their experimental materials adequate and their interpretation of results correct? Here Yoshida's Sarcoma, which is possessed of the foregoing suitable quality, is believed to be the most promising material for the pursuit of these kinds of immunological phenomena. As a matter of fact, Takeda (10) and his cooperators have already studied with this sarcoma the phenomena of

immunity and allergy in regard to tumor cells. They showed that conspicuous immunological phenomena were observed in the transplantation of tumors.

If such conspicuous immunological phenomena are also observed among malignant tumors, they will positively play an important part in the studies of diagnosis and treatment. With this in view I investigated immunological facts, using Yoshida Sarcoma. My aim was to disclose most accurately their qualities and intensity if there be any such fact. The experiments I carried out are divided as follows:—

1. Experiment of immunity in re-transplantation. 2. Experiment on effect of tumor vaccine. 3. Experiment of passive immunity. 4. Agglutination of tumor cells. 5. Precipitation by tumor extract. 6. Cutaneous reaction by tumor extract.

Procedure of Research

1. Experiment of immunity in re-transplantation.

Hypodermic transplantation of 0.02 to 0.03 cc of tumor ascites was made in the back of each animal beforehand and after various periods of time—for instance, five days for one animal and seven days for another—0.01 to 0.02 cc of ascites was transplanted again in the peritoneal cavity of the same animal. The earliest re-transplantation was carried out on the very same day of the first transplantation and the latest on the fourteenth day. Immediately before each re-transplantation, ascites of the examined animal underwent microscopic observation in Giemsa-stained smear to make sure that there was no metastatic tumor cell coming from the transplanted part of the back. After re-transplantation microscopic observation of ascites was taken every day to examine the increasing process of tumor cells in the peritoneal cavity, and further, the growing condition of hypodermically transplanted tumors was observed.

The result was as follows: If re-transplantation was on the first to third day from the hypodermic transplantation, the tumor cells in the peritoneal cavity increased to the state of pure culture in three days or so, making little difference in their growing condition from ordinary cases. But when the second transplantation was carried out on the fourth to sixth day from the first one the increase to that state was a little retarded—it required six to seven days. Re-transplantation on and after the seventh day did not allow tumor cells to proliferate to that state till immediately before the death of the tumor animals or never allowed such active increase to the end (Table 1). In other words, the growth of re-transplanted tumors was clearly checked, but the power was not so strong as to prevent the tumor cells from increasing- Furthermore, the following facts were observed. In all the cases of re-transplantation, emigration of leucocytes activity

Table 1. Growing process of tumors implanted hypodermically and intraperitoneally in the re-transplantation experiments with Yoshida Sarcoma.

Tumors re-transplanted intraperitoneally				Tumors transplanted hypodermically		
Date of re-trans.	Number failure in parenthese	Days of pure cultur state in peritoneal cavity	Duration of existence of leucocyte	Days from re-trans. to death.	Magnitude at time of re-trans.	Magnitude at time of death
1	1	4	Hardly any seen	11	not palpable	Cherry-sized
2	1	4	〃 after the 4th day	12	〃	〃
3	2	4	〃 〃	7, 6	Pea-sized	〃
4	1	5	Hardly any seen after the 7-8th day	10	〃	〃
5	3 (1)	5, others 7	〃 〃	6, 11	Bean-sized	Walnut-sized
6	2 (1)	7 8	〃 〃	8	〃	〃
7	4 (2)	7, others (−)	Observed to the last	10, 10	Cherry-sized	〃
8	2 (1)	(−)	〃 〃	8	〃	〃
9	4 (2)	7, others spontaneously cured			〃	
10	4 (3)	(−)	Observed to the last	10	〃	Walnut-sized
11	1	8	〃 〃	12	Bean-sized	〃
12	1	(−)	〃 〃	14	Cherry-sized	4 x 2.5 cm
13	2 (1)	9	〃 〃	11	Walnut-sized	8 x 5 cm
14	3 (1)	(−)	〃 〃	8, 9	〃	6 x 6 cm

Remarks: 1. Amounts of implanted tumors: 0.02–0.03 cc. hypodermically, 0.01–0.02 cc. intraperitoneally.

2. Magnitude of tumor: Pea-size.....0.6 cm. diam.
Bean-size .....0.1 cm. diam.
Cherry-size .....2 cm. diam.
Walnut-size .....3 cm. diam.

3. (−) means that tumor cells have not yet reached the pure culture state, though showing proliferation in peritoneal cavity.

4. Animals used weighed 100 gr. or so.

continued longer than in ordinary transplantation. In this latter case leucocytes usually disappear after twenty-four hours, though for several hours following the transplantation they make their active appearance. But, in my experiments when re-transplantation was made on and after the seventh day from hypodermic transplantation, I had even such a case in which powerful leucocyte reactions were seen to the very last. It may be said that this fact corroborates the opinion Takeda has held that the allergy phenomenon is also noticed in tumor trans-

plantation. Upon the growing process of hypodermically implanted tnmors any remarkable influence of intraperitoneal re-transplantation could not be observed.

2. Experiment on effect of tumor vaccine.

Before tumor transplantation intraperitoneally or for days together after it 0.5 to 1 cc of tumor vaccine was injected in the peritoneal cavities of rats about 100 g in weight. This vaccine was made by diluting tumor ascites (500,000 to 700,000 tumor cells included in a cubic mm) twelvefold or sixfold with physiologic NaCl solution (0.5% of carbol added) and then heating it in a waterbath at 53°C for an hour (Experiments 1, 2) or in an incubator at 38°C for twenty-four hours (Experiments 3, 4); after that it was stored in a refrigerator. Before the main experiments were begun, 0.5 cc of this vaccine was preliminarily inoculated into the peritoneal cavity of each of ten untransplanted rats and the process was observed for two weeks to make sure of the death of all the tumor cells. For control physiologic solution of NaCl with 0.5% of carbol added was employed.

The expected results were not attained. In the first experiment, a 1/12 dilute vaccine was given for a week and then tumor ascites was transplanted five days after the last injection. In the second experiment, the first injection of a 1/6 dilute vaccine was begun at the same time of the transplantation. Injections were continued for a week. The third experiment was to inject a 1/6 vaccine for two weeks before ascites transplantation. With the same vaccine I tried the fourth experiment in which injection was carried on every other day for thirteen days and the transplantation was made ten days after the last injection. In each case there was no difference from the control experiment and, though some animals had feeble leucocytes reaction to the last stage, the vaccine did not prolong their lives (Table 2). In other words, lifeless cells failed to bring about any prophylactic effect.

3. Experiment of passive immunity.

The transplantability of Yoshida Sarcoma is about 98%. The remaining 2% of animals are constitutionally insusceptible to this sarcoma. Even these animals, however, when tumors are transplanted, permit a temporary proliferation of cells, which, afterwards, show a rapid decrease, ceasing to be about ten days after. This may be looked upon as a spontaneous healing. Therefore I tried to examine how the sera from such animals as of this nature would act on tumor cells in test-tubes.

In the peritoneal cavity of each of four spontaneously cured animals, re-transplantation was made a month after the cure. One, two, and one were killed on the second, twelfth and twenty-eighth days respectively from the re-transplantation in order to obtain sera. 0.1 cc of tumor ascites was added to 1 cc of each of

Table 2

Effects of tumor vaccine on Yoshida Sarcoma transplanted.

Exp. 1.

No. of animal	weight	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
3583	85 gr	○ ○
3584	100 "	○ X
3585	90 "	● X
3586	65 "	● Died of Pneumonia

Exp. 2.

No. of animal	weight	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
3590	85 gr	○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○
3591	110 "	○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ X
3592	85 "	● ● ● ● ● ● ● ● ● ● ● ● ● ● ● ● ● ● ● X

Exp. 3.

No. of animal	weight	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
3606	110 gr	○ X
3607	130 "	○ X
3608	120 "	● X
3609	110 "	● X

Exp. 4.

No. of animal	weight	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13---23 24 25 26 27 28 29 30 31 32 33 34 35
3610	100 gr	○ X
3611	90 "	○ X
3612	90 "	● X

Remarks: ○ *Vaccine injected*

● *Physiologic NaCl solution (carbol added) injected (for control)*

——X *from transplantation to death*

↑ *tumor transplanted*

the sera in test-tubes, which were stored in an incubator at the temperature of 37°C. Six hours, twelve hours and twenty-four hours after this 0.2 cc of one of these mixtures was inoculated into the peritoneal cavity of an untransplanted rat. So with other three mixtures. For control sera obtained from untransplanted normal rats were used.

These experiments revealed that the sera of the second and the twenty-eighth day allowed the tumors to grow without any difference from control tests, while the sera of the twelfth day, both of them, caused the tumors' growth to be later by two to twelve days than in control tests (Table 3). Further, the experiment of injecting sera from spontaneously cured animals into the peritoneal cavities of tumor animals was tried. In this case the injection of 3 cc of sera brought about, immediately after, a moderate degree of change in tumor cells, but on the very next day the cells showed again an active proliferation. From this it is known that when transplantation is tried into spontaneously cured animal, in the sera, after having been cured, the animal has a checking factor (antibody) against tumors, though this factor will diminish its action in a month or so. Even in this case the power is not so strong as to destory the implanted tumor cells.

4. Agglutination of tumor cells.

There has been no report published that the agglutination was attained by sera from tumor persons or animals. I think this is because all the tumors we knew were nodular ones, with which it is technically difficult to pursue such a phenomenon. With Yoshida Sarcoma I succeeded in finding the fact that there arose an agglutination peculiar to these tumor cells by the following method:

The process I took is: Animals were bled from the heart and the serum obtained. This bleeding was made without killing them and that several times in various conditions of one and the same animal before and after transplantation. For antigen thick tumor ascites a week after transplantation was taken out and by washing it several times in physiologic NaCl solution was made a fluid with nothing else but cells suspended in it. Now my experimental method is: first of all, sera are diluted to various concentrations, then on object glasses antigen is added to each of these sera of different dilutions. This is stirred and mixed uniformly, care being taken that the cells are not piled one on another by microscopic examination. After twenty minutes in an incubator at 37°C, and then three hours at room temperature, microscopic observations are made under a low magnification.

The result was as follows: when the reaction was negative each of the cells lay down still at the bottom of the fluid at a certain interval, but when positive the agglutinated cells lay scattered, several scores or hundreds of them forming separate clumps (see Photos). By this method it was found that the titer of agglutination came to be 1: 100 to 1: 200 after the tenth day from transplantation (Table 4). But the tests in normal animals gave positive reactions at 1: 10 to 1: 25, sometimes 1: 50. This proves that normal animals have also a certain degree, though low, of agglutinin, which is made to increase by the transplantation of tumors. In this connection I tried experiments with the supposition that

Table 3.

Effects of serun from spontaneously cured animals on Yoshida Sarcoma.

Exp. 1. (Serum obtained on 2nd day from transplantation)

No. of animal	Hours stored in incubator	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
3627	6	——————————X
3628	"	- - - - - - - - - - - - - X
3629	12	————————————————————————————⁜
3630	"	- X
3631	24	——————————————————————X
3632	"	- X

Exp. 2 (Serum obtained on 12th day from transplantation)

No. of animal	Hours stored in incubator	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
3616	6	————————————————————————X
3617	"	- - - - - - - - - - - - - X
3618	12	——————————————————————X
3619	"	- - - - - - - - - - - - - - - - - X
3620	24	————————————————————————————X
3621	"	- - - - - X (Died of Pneumonia)

Exp. 3. (Serum obtained on 12th day from transplantation)

No. of animal	Hours stored in incubator	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
3654	6	——————————————————X
3655	"	- - - - - - - - - - - - - - - - - X
3656	12	————————————————————————X
3657	"	- - - - - - - - - - - - - - - - - - X
3658	24	————————————————————————X
3659	"	- X

Exp. 4. (Serum obtained on 28th day from transplantation)

No. of animal	Hours stored in incubator	Days after transplantation 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
3646	6	——————————————————X
3647	"	- X
3648	12	——————————————————X
3649	"	- - - - - - - - - - - - - - - - X
3650	24	——————————————————X
3651	"	- X

Remarks ——— *Cases in which serum from cured animals was employed*
- - - - - *Cases in which serum from untransplanted animals was employed (for control)*
⁜ *Spontaneously cured*
All animals weight 85 to 150 gr.

Table 4.

Agglutination of Yoshida Sarcoma by the sera of rats 80 to 120 gr. in weight.

Days	Number	Classification by agglutination titers	Dilution of serum 0 x 10 x 25 x 50 x 100 x 200 x 400 x
Before transplantation	5	2	+
		2	+
		1	+
4th day from transplant. (intraperitoneally)	3	2	+
		1	+
6th day (")	3	3	+
8th day (")	3	2	+
		1	+
10th day (")	4	3	+
		1	+
12th day (")	2	2	+
15th day (hypodermically)	1	1	+
18th day (")	2	2	+

spontaneously cured rats, a small number of which were among albino rats we were using, might have constitutionally a high titer of agglutination. But this fact was not established. In other words, between transplantable and untransplantable animals there can be found no difference in the titer of agglutination they have by nature. My investigation of the changing conditions of the agglutination titers in spontaneously cured rats made it known that the rat having 1 : 25 of the titer before transplantation showed 1 : 100 after the treatment, which condition continued for about a month after the tumor had been spontaneously healed. Then the original titer was regained. (Table 5)

Next I took rabbits and guineapigs as heterogenous animals and investigated their normal agglutination titers. They all gave the titer of 1 : 100. We may know from this that heterogenous animals have a much higher titer of normal agglutination than rats. In the further experimental tests with guinea pigs, a dilution as high as 1 : 800 could be reached by repeating transplantation. No phenomenon of increase of titer was observed with the serum sensitized with normal cells such as liver cells or red cells.

5. Precipitation by tumor extract.

When tumor extract which had been obtained by using distillated water was

employed for antigen, positive reactions, though in a low degree, were observed. But the filtrate of this extract after heated or supernatant tumor ascites used for antigen made the test end in negative results.

6. Cutaneous reactions by tumor extract.

Yoshida Sarcoma was extracted by ethyl alcohol and the extract was condensed to 1/5 in volume and this condensed extract was used for the examination of cutaneous reaction. When normal animals received an injection of this extract in amounts of 0.1 cc they equally took on a heavy red color with callosity around the pallid spot of the injected part after 1 to 2 hours. This color was less distinct in animals which had passed four or five days after transplantation. If the injection was made into animals six days or more after transplantation there could be seen no such coloring. Mere injection of alcohol for control made a very weak presentation both of the callosity and of coloring as compared with the foregoing cases. Following these experiments, further experiments were tried, with this sarcoma, on Botelho reaction, (11) Kürten reaction (12) and Shichijo reaction (13). The results were that positive signs were displayed on the tenth and the eighth day after transplantation in Botelho reaction and Kürten reaction, respectively. In Shichijo reaction, though the complete vanishing of arabesque was on the tenth day, considerably different figure from normal cases was noted even previous to that. Comparison of these phenomena makes me conclude that cutaneous reaction appears comparatively soon though there is no such observation whatever in the earliest period after transplantation.

Apart from the previous studies I made investigations, with a view to contributing to diagnosis, on the changing amounts of proteins in sera and of blood sugar after the transplantation of Yoshida Sarcoma. The amount of serum-proteins only took a gradualy decreasing course, but as to the change of amount of blood sugar a fairly interesting result was obtained (Table 6). The amount began to rise on the second day after transplantation, reached its height on the fourth day, and then showed a gradual fall, becoming lower than in normal conditions on the eighth day. The fourth day when the largest amount of blood sugar was reached corresponds with the period in which the tumors showed the most decided increase in numbers by active division, just immediately after entering the state of pure culture. This measurement was conducted with the collaboration of T. Yagi.

Sugimoto's (14) statement, which was given after examining cases of stomach cancer, that tumors in the early stage should generally present hyperglycemia, but that then gradually they should change to hypoglycemia, was confirmed by our examination results. That is, the amount of blood sugar is known to be very huge in the early stage when cutaneous reaction is in uncertain positivity.

Table 5

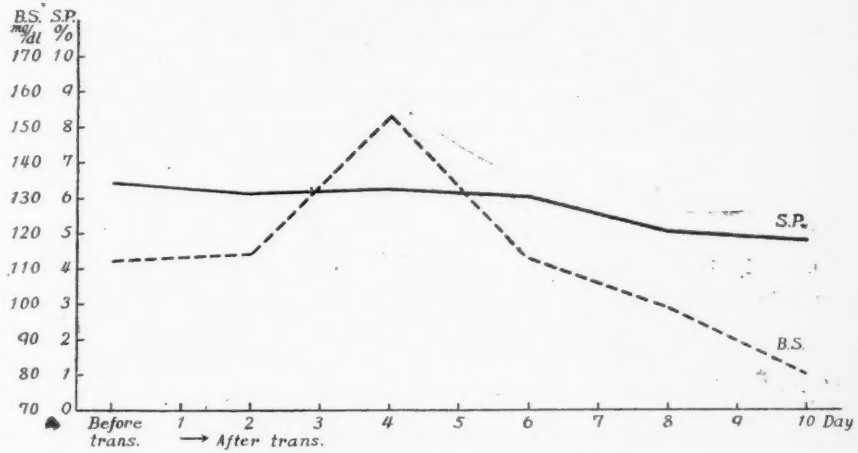
Agglutination of Yoshida Sarcoma by the Sera of rats 160 to 230 gr. in weight.

No. of animal	Before trans.	Days after transplantion																				
		2	4	6	8	10	12	14	16	18	20	22	24	26...	30...	34...	38	40...	45	50...	60...	70... 80
X_1	25 X		25 X			100 X		(Died)														
X_2	50 X			50 X		200 X		200 X			(Died)											
X_3	25 X			50 X		(Died)																
X_4	25 X			50 X		200 X		(Died)														
X_5	25 X			50 X		(Died)																
X_6	25 X			50 X		100 X		100 X					50 X				25X*					
X_7					50 X			100 X			(Died)											
X_8	50 X								200 X				200 X		(Died)							
X_9	25 X					100 X			(Died)													
N_1								100 X				100 X				50 X		25X*				
N_2										100 X										100 X	50 X	50X*

Remarks: N means rats in which tumors were transplanted a month before and which were, it was confirmed, cured spontaneously afterwards.
* spontaneously cured.
Tumor were all inoculated intraperitoneally.

Table 6.

Change of amounts of serum-proteins and of blood sugar (average value of 36 cases). Proteins were measured with Pulfrich's Reflactmeter. Sugar by Hagedorn-Jensen's method.



Discussion and Summary

1. Of immunological phenomena of malignant tumors, I have obseved, by satisfactorily exact experimental methods, the following six cases: immunity in re-transplantation, effects of tumor vaccine, passive immunity, agglutination, precipitation, and cutaneous reaction; and found it clear that antibodies to tumor cells are produced by transplanting tumor cells. Production of antibodies was increased to some degree along with the propagation of tumors, but the productivity was not so strong as seen in the bacteriological field. The most distinctive fact I noticed was the agglutination of tumor cells.

Eaton (15), who tested agglutination of Plasmodium knowlesi by immune serum from chronically infected and superinfected monkeys, reported that the titers of agglutination were given at dilutions of only 1: 16 to 1: 64 $\pm$ in the acute stages and at dilutions of 1: 256 to 1: 1,024$\pm$ in the chronic stages or after some ten infections of this disease were received.

Agglutinin that operates upon tumor cells is also found, in a small amount (about 1: 25), even in normal animals. This gradually increases (1: 200) with tumor cells implanted, but not so by the sensitization with other cells, for instance, liver cells or red cells. Therefore I may safely say that this phenmenon is specially confined to tumor cells.

Tumor vaccine forms a subject of interesting study. Successive inoculations of this vaccine were carried out in diverse ways before and after the transplantation

of tumors, but the growth of tumors could not be restrained.

In regard to the effects of serum of cured animals, Takeda (16) and his co-operators reported that they transplanted Yoshida Sarcoma in successive generations of white rats of Wister-strain and with 0.5cc of serum from these spontaneously cured animals they were successful in completely preventing tumors from proliferation.

In my experiments serum from cured animals proved effective to some degree in retarding the proliferation of implanted tumors, but not so powerfully as to hinder it.

2. I investigated a cutaneous reaction which was considered as neutralizing phenomenon of tumor toxin.

Recently considerable attention has been paid to such reactions (17) as diagnostic methods of cancer and these methods have a much higher positivity as compared with other means of diagnosis. My method of reaction can be said to be a new one as to obtaining an antigen. Certainly this reaction was produced sooner than several other diagnostic methods I tried. but could not be expected in the early period after transplantation. In other words, even such a reaction requires a certain time in letting the body respond to it. Here lies difficulty of diagnosis of cancer. On the other hand, however, there is a fact that blood sugar increases in amounts describing a certain curve in the early stage after transplantation. With this fact into consideration the decision of positivity may be hastened by judging from cutaneous reaction and blood sugar titer together. That is, in the early period in case the blood sugar titer is high, the cutaneous reaction may safely be judged as certain positive, even though it may by itself be apparently uncertain positive.

Conclusion

1. The experiments with Yoshida Sarcoma clearly made it confirmed that immunological phenomena are observed in the transplantation of malignant tumors.

However, the immunity by itself, does not show so powerful an effect in the prophylaxis or cure transplanted tumors.

2. The value of cutaneous reaction with tumor extract to be employed for diagnosis in the early period can be heightened by taking the blood sugar titer in consideration.

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* Written in Japanese.

要　旨

悪性腫瘍に於ける免疫学的現象の研究
(吉田肉腫による実験的研究)

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悪性腫瘍における免疫学的現象の観察に最も適当な吉田肉腫を用いて，次の諸項目に就て実験を行つた。

1. 再移植免疫の実験；第1回の移植により，第2回の移植はその成長を或程度遅延させるが，完全に抑制される事実は認めることは出来ない。

2. 腫瘍細胞ワクチンの効果に関する実験；移植された腫瘍の発育に対して抑制的には作用を示さない。

3. 被動性免疫の実験；自然治癒した動物の血清を用いて，体外で腫瘍細胞に混じても，又は移植後に注入しても腫瘍の発育を著しく阻害することは出来ない。

4. 腫瘍細胞の凝集反應；腫瘍動物の血清中には腫瘍細胞に対する凝集素が明に増加することを認める。この現象は今回の実験中最も顕著な事実に属する。

5. 腫瘍細胞抽出液による沈降反應；腫瘍細胞の蒸溜水エキスを腫瘍動物の血清に加えると微弱ながら反應陽性を認める。

6. 皮内反應；腫瘍細胞のアルコールエキスを用いて腫瘍動物に1種の皮内反應を発現させることが出来る。反應は移植後6日目以後において最も著明である。

以上の実験成績から，腫瘍細胞を移植することにより，腫瘍細胞に対する抗体が作られることは明である。そして，抗体は腫瘍の増殖と共に或程度増加することも認められる。併し，その強さは細菌学領域における様に著しいものでは無いと言わねばならない。

尚ほ，吉田肉腫動物では移植後血糖價が一定の曲線を以て推移し，4日目にはその頂点を示す。この時期には皮内反應は不確実陽性であるが，血糖價と合せて判定すれば，この反應を確実陽性と認めることが出来る。

人癌でも，早期においては概して血糖價の上昇を示す場合が多いと報告されておるから，人体の場合にも皮内反應と同時に血糖價を考慮に入れれば，この反應の判定をより早期に，且つ確実にするだろうと考える。

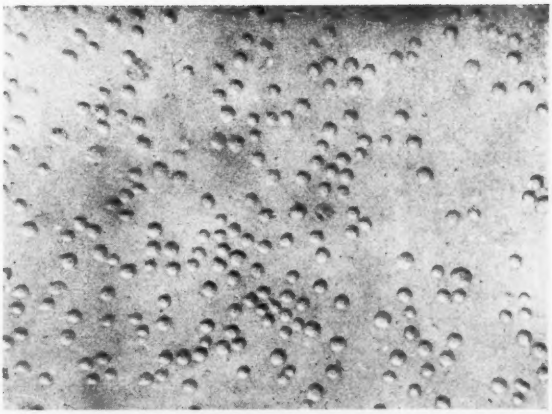

Fig. 1.
Agglutination, negative.

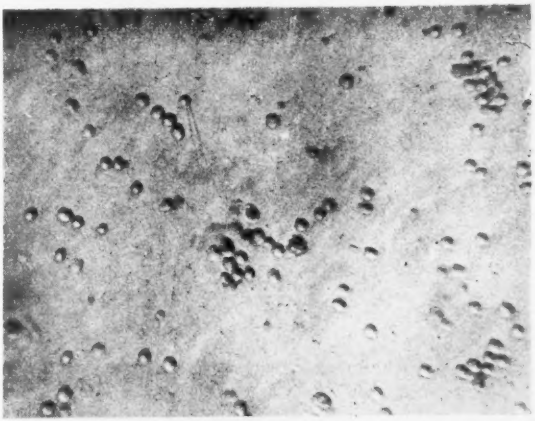

Fig. 2.
Agglutination, positive
(weak).

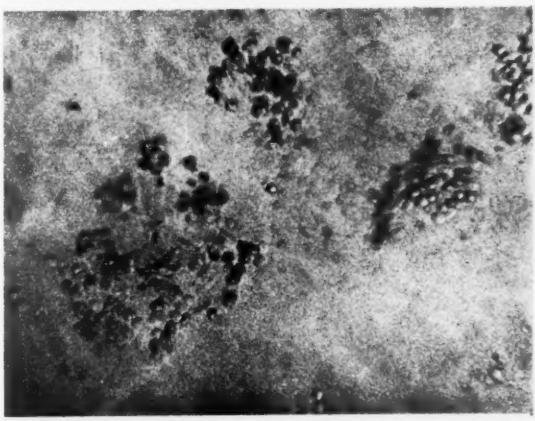

Fig. 3.
Agglutination, positive
(strong).

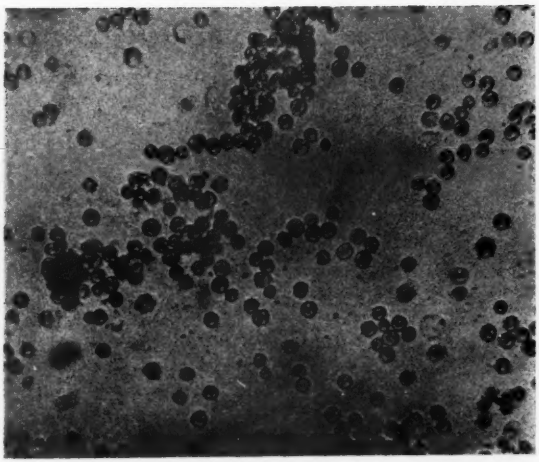

Fig. 4. Clumps of cells in weak positive agglutination (under phasemircoscope)

Fig. 5. Clumps of cells in strong positive agglutination (under phasemicroscope)

[GANN, Vol. 42; March, 1951]

THE SPECIFIC GRAVITY OF THE TISSUES OF RATS FED P-DIMETHYLAMINOAZOBENZENE, WITH A NOTE ON THE SPECIFIC GRAVITY OF THE TISSUES OF TUMOR MICE*

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An attempt to estimate the specific gravity of the liver of rats fed p-dimethylaminoazobenzene (butter yellow) was reported in the previous paper.[1] The purpose of this paper is to present the specific gravity of liver and other tissues of rats during the course of liver cancer production by the same azo-compound.

METHODS

For the determination of the specific gravity copper sulphate method which was introduced by Phillips, van Slyke and other co-workers[2] and thereafter was adapted to the measurement of the specific gravity of tissues by Opie,[3] was used. In Japan, the copper sulphate method was introduced by Yoshikawa[4] and adapted to the animal tissues by Nawa et al.[5] Small pieces of liver or other tissues, just half the size of a rice grain, were inserted below the surface of the solutions with graded specific gravity, namely, a series of copper sulphate solution in quantities of 100 cc varying in specific gravity from 1.040 to 1.100 and differing in succession by 2 units. Standardized hydrometers have been used to test the specific gravity of the solutions that have been used and to determine when they have become altered by repeated use.

A group of normal adult albino rats, male and female, was maintained on the usual butter yellow rice diet (0.6 g butter yellow in cod liver oil per 1 kg of polished rice) so as to produce liver cancer.[6] At various periods in the course of the feeding experiment, a suitable number of the male and female rats were taken at random and the specific gravity of the liver and other tissues (spleen, kidney, muscle and brain) was determined according to the cupper sulphate method as described above.

In expressing the nature of the liver findings, four grades of the pathological changes leading to the production of liver cancer were indicated as macroscopically normal, uneven surface with but slight connective tissue proliferation, cirrhotic liver and liver cancer. The identification of these liver findings has been described

* Aided by a Scientific Research Encouragement Grant from the Departmeet of Education.

in detail in the publication cited.[7]

Besides, a group of normal albino rats, male and female, was maintained on the diet of polished rice alone and these rats were used as controls.

Experiments

At first, the specific gravity of the tissues of normal rats was estimated based on both sexes of 20 specimens each. The average value of the specific gravity of the tissues obtained are presented in Table 1. It is noticeable that each average of the specific gravity of each tissue of the male rats is always higher than female, in the degree of 0.001 to 0.004. Especially, the difference of the specific gravity of the liver between male and female rat is respectable. That is, the average specific gravity of the liver of the male rat is 1.088, while in the female 1.084.

Thereafter, the estimation of the specific gravity of the tissues of the rats fed butter yellow was made.

And the average values of these results are summarized in the same table (Table 1) according to their liver findings: macroscopically normal, uneven surface, liver cirrhotic and cancer. These averages change in the specific gravity of tissues of rats fed butter yellow in Table 1 were plotted in Chart 1. It will be seen clearly from the table and the chart that the production of liver cancer is associated with the marked decrease in the specific gravity of the liver as previously reported. Besides, the moderate increase of the specific gravity of the spleen is demonstrated. In the cases of the kidney and muscle, less changes than in the two tissues described above are to be seen. And in the case of brain, little variation in the specific gravity is found.

It is noteworthy that these changes in the specific gravity are most marked in the liver and are moderate in the spleen in which the remarkable pathological lesions were demonstrated during the experimental production of liver cancer by butter yellow feeding by many investigators.

In the following table (Table 2), the water content of the liver and spleen is presented. In this table, it will be seen that the water content increases in the liver tissues, and decreases in the spleen in the process of liver cancer production. These results in the water contents of the tissues are similar to those of the previous investigators.[8]

Furthermore, it is reported that the lipid content increases in the liver and decreases in the spleen during the course of liver cancer production by Masayama,[9] Iki,[10] and Fujiwara, Nakahara and Kishi.[11] Therefore, it appears that the increase and decrease of the specific gravity of the tissues are closely related to the content of water and lipids in the tissues of rats in the process of liver cancer production.

Table 1. The Average Specific Gravity of the Tissues of Rats during the Course of Liver Cancer Production.
(The minimum and maximum values in the brackets)

Liver Findings	Sex	No. of Rats	Specific Gravity				
			Liver	Spleen	Kidney	Muscle	Brain
Controls	♂	20	(1.083-1.094) 1.088	(1.066-1.074) 1.071	(1.060-1.072) 1.065	(1.064-1.076) 1.071	(1.047-1.051) 1.050
	♀	20	(1.077-1.090) 1.084	(1.065-1.074) 1.069	(1.057-1.067) 1.063	(1.061-1.075) 1.070	(1.043-1.050) 1.048
Macroscopically normal	♂	11	(1.068-1.089) 1.077	(1.069-1.076) 1.072	(1.064-1.073) 1.068	(1.057-1.075) 1.067	(1.044-1.051) 1.048
	♀	6	(1.066-1.087) 1.077	(1.071-1.078) 1.075	(1.066-1.071) 1.068	(1.070-1.076) 1.073	(1.047-1.049) 1.048
Uneven surface	♂	6	(1.066-1.075) 1.070	(1.072-1.074) 1.073	(1.065-1.068) 1.066	(1.070-1.079) 1.073	(1.047-1.050) 1.048
	♀	6	(1.070-1.079) 1.074	(1.070-1.077) 1.073	(1.066-1.069) 1.067	(1.065-1.074) 1.073	(1.047-1.050) 1.049
Liver cirrhosis	♀	3	(1.057-1.075) 1.067	(1.072-1.079) 1.076	(1.060-1.066) 1.064	(1.068-1.070) 1.069	(1.051-1.052) 1.051
Liver cancer	♀	5	(1.054-1.067) 1.060	(1.075-1.077) 1.077	(1.066-1.070) 1.067	(1.062-1.067) 1.065	(1.046-1.051) 1.049

Table 2. The Average Value of the Water Contents of the Liver and Spleen of Rats during the Course of Liver Cancer Production.

Liver findings	Sex	No. of Rats	Water Contents (%)	
			Liver	Spleen
Controls	♂	13	70.0	74.2
	♀	11	68.8	73.7
Macroscopically Normal	♂	6	74.1	73.9
	♀	6	73.6	74.8
Uneven Surface	♂	5	70.8	74.7
	♀	6	71.6	74.6
Liver cirrhosis	♂	2	72.0	74.3
	♀	3	74.6	—
Liver cancer	♀	3	77.6	72.6

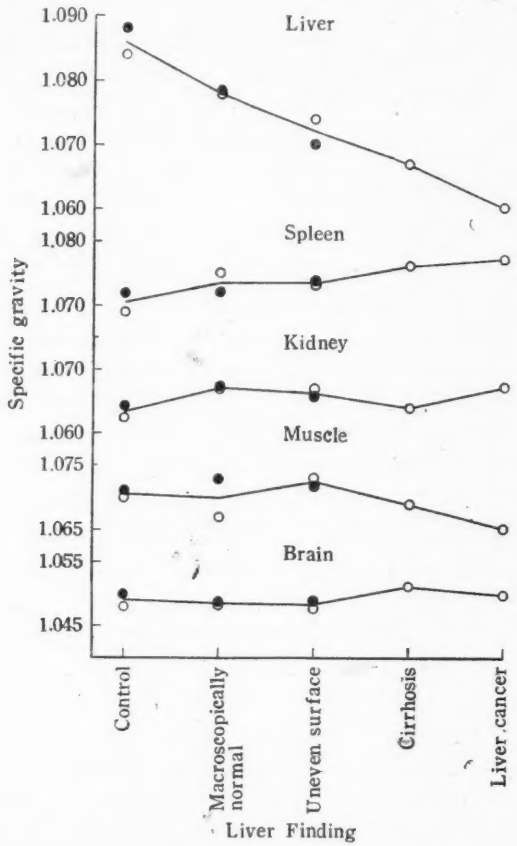
Summary and Conclusion

The specific gravity of the liver, spleen, kidney, muscle and brain of the rats fed p-dimethylaminoazobenzene was determined by the copper sulphate method. And it was shown that the changes in the specific gravity are most remarkable in the liver and spleen in the course of liver cancer production. The specific gravity of liver decreases strikingly, and that of spleen increases slightly. A

Chart 1.

The Average Change in the Specific Gravity of the Liver, Spleen, Kidney, Muscle and Brain in the Course of the Production of Liver Cancer.

Each circle indicates an average specific gravity of females; the solid circle that of males.



little variation in the specific gravity of the kidney and muscle was found. In the case of the brain however, the specific gravity was unchanged throughout the experiment. It appears that these variations in the specific gravity of the tissue of rats in the process of cancer production are closely related to the content of water and lipids in the tissue.

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A Note on the Specific Gravity of the Tissues of Tumor Mice.

In connection with the changes in the specific gravity of the tissues of rats during the course of the liver cancer production by p-dimethylaminoazobenzene presented above, demonstrating a striking decrease of the specific gravity of the liver and a moderate increase of that of the spleen, it is here reported that the changes in the specific gravity of the tissues of mice were detected after tumor transplantation.

The tumor (S 150 Strain), a spindle cell sarcoma, was originally produced by Takizawa[2] in a mouse by daily injections of fructose, propagated in generations of mice, and now the transplantability became fairly well fixed (Takizawa, N. GANN, Vol. 32, 236, 1938). The rate of positive transplantations of this sarcoma is almost 100%, and the growth rate of the tumor is very rapid, the swelling of the tumor reaching the maximum 2x3x4 cm^3 in size usually in about two weeks after transplantation. Very often, the tumor perforated through the skin at the end of this period. The transplantation was made by subcutaneously implanting into mice small fragments of the tumor in the usual method.

The tumor mice were killed by exsanguination at the periods of 1, 3, 5, 10 and 15 days after transplantation, very small slices were cut from liver, spleen, kidney, muscle and brain. And their specific gravities were estimated by the copper sulphate method as previously described.[3)4)]

The specific gravity of Takizawa's sarcoma tissues was 1.053–1.055 and that of the necrotic parts 1.045–1.046.

The average values of the tissue specific gravity of tumor mice are summarized in Table 3. The average specific gravity of the liver of the normal mouse was 1.081, while that of the tumor mouse was 1.073 just 1 day after the transplantation. Thereafter, the average specific gravity of the liver maintained on the degree of 1.073, on 3 or 5 days after the sarcoma graft took positive. At 10 to 15 day period, the average specific gravity of the liver of tumor mouse decreased to the range of 1.070 or 1.066 respectively.

In the case of the spleen, the average specific gravity of normal mice was 1.071. At 15 days after the tumor transplantation, the specific gravity of the spleen reached 1.069. In other three tissues, the same tendency of very slight decrease in the specific gravity was demonstrated. In a word, the specific gravity of each tissue was reduced according to the growth of the established graft.

In the mice in which the graft showed a slight temporary growth and subsided soon later, the specific gravity of each tissue was less than the normal, but higher than in the sarcoma mice.

The specific gravity of the spleen run counter to that of the liver of rat during the course of liver cancer production by butter yellow, but in tumor mice the specific gravity of the spleen does not increase but decrease slightly.

Table 3. The Average Specific Gravity of the Tissues of Tumor Mice.
(The minimum and maximum values are in the brackets.)

Exper. days	No. of mice	Sex	Specific Gravity				
			Liver	Spleen	Kidney	Muscle	Brain
Controls	10	♀	(1.080-1.082) 1.081	(1.069-1.075) 1.071	(1.058-1.065) 1.062	(1.063-1.076) 1.071	(1.051-1.051) 1.051
1	5	〃	(1.071-1.075) 1.073	(1.069-1.071) 1.070	(1.060-1.065) 1.062	(1.070-1.075) 1.072	(1.048-1.050) 1.049
3	7	〃	(1.065-1.075) 1.073	(1.068-1.070) 1.069	(1.057-1.067) 1.058	(1.065-1.075) 1.070	(1.047-1.052) 1.049
5	7	〃	(1.072-1.077) 1.073	(1.067-1.071) 1.069	(1.057-1.067) 1.060	(1.067-1.077) 1.072	(1.045-1.051) 1.049
10	15	〃	(1.059-1.075) 1.070	(1.065-1.069) 1.067	(1.055-1.065) 1.059	(1.065-1.077) 1.070	(1.046-1.053) 1.049
15	15	〃	(1.061-1.071) 1.066	(1.065-1.070) 1.067	(1.055-1.065) 1.059	(1.064-1.076) 1.070	(1.047-1.054) 1.050
20-25*	8	〃	(1.073-1.079) 1.077	(1.066-1.071) 1.069	(1.057-1.065) 1.060	(1.070-1.074) 1.071	(1.048-1.050) 1.050

*Mice in which the sarcoma grafts failed to take.

The writers are greatly indebted to Prof. Takizawa, Chiba Medical College, for his kindness in sending the sarcoma.

抄　　錄

実驗的肝癌生成過程に於ける白鼠の臟器比重
附. 肉腫移植廿日鼠の臟器比重に就て

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木下法に従い，白鼠に p-Dimethylaminoazobenzene を飼與して，実驗的に肝癌を生成する過程において，肝，脾，腎，筋及び腦等の各臟器の比重を，硫酸銅法を用いて測定した。

実驗に先立ち，正常白鼠の臟器比重を測定したところ，上記のいずれの臟器においてもその比重は雄鼠の方が雌鼠よりいくらか高い値を示した。而してこれらの差は肝比重では最も顯著で，雄鼠の肝の平均比重 1.088 なるに対して，雌鼠では 1.084 であつた。実驗日数に従つて白鼠を出血死せしめ，先ずその肝所見に應じて肉眼的正常，表面不平滑，肝硬変及び肝癌の 4 群に動物を分ち，夫々の臟器比重を測り比較検討した。

これらの臟器の中では肝比重が最も著しい変化を示した。即ち肝比重は，正常鼠では雄1.088 雌 1.084 を示したのが，肝所見の進行と共に雌雄の別なく階段的に下降して，硬変を示す肝では 1.067 を，肝癌組織では遂に 1.060 を示すに至つた。肝に次いでかなりの変化を示したのは脾であつて，その比重の値は肝と反対に肝の病変の進行に伴つて軽度ではあるが上昇した。尙軽度ではあるが腎比重は上昇，筋肉比重は下降の傾向を示している。最後に腦比重は殆ど不変であつた。

一方，これら肝及び脾の含水量の測定を行い，水分が実驗の経過に應じて肝では増加，脾では減少の傾向のある事を確めた。これらの結果を類脂体の含有量に関する諸研究者の報告と併せ考える時，肝癌生成過程における臟器比重の変化は，これら日を追うて増加或は減少を示す臟器の水分並びに類脂体の含量に，大いに関係あるものと考える。

尙ほ上記の実驗的肝癌生成過程における大黒鼠の臟器殊に肝並びに脾の比重が実驗の進行と共に著しく変化することと関連して，別に移植性肉腫廿日鼠における臟器比重を検討した。移植性肉腫としては千葉医大滝沢教授が濃厚果糖溶液の反覆注射により生成した繊維肉腫を用いた。この肉腫の移植率は大体 100%陽性で移植片は速かに増殖し平均約 2 週間前後で 2×3×4cm³ の大きさに迄達し，ために宿主を死に到らしめる。実驗は移植針を用いて皮下移植された廿日鼠を移植後 1 日，3 日，5 日，10 日，並びに 15 日目に出血死せしめ，その臟器小片を切りとり，硫酸銅法を用いて比重を測定した。肝，脾，腎，筋並びに腦の比重を日を追うてし

らべた結果，特に肝比重が著しく減少する事がわかつた。他の臓器比重も軽度ではあるが，肉腫の増殖するにつれて，いずれも減少した。又移植陰性を示した動物の臓器比重は正常鼠と肉腫鼠との中間値を示した。

（文部省科学研究費による）

[GANN, Vol. 42; March, 1951]

ARGININE CONTENT IN THE TISSUES OF RATS FED P-DIMETHYLAMINOAZOBENZENE*

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The estimation of protein or amino acids in the liver of rats fed p-dimethylaminoazobenzene (butter yellow) is not only of general biochemical interest, but is also worthy of special attention in the study of the relation between carcinogenesis and physiological conditions of liver. Nakano[1] has reported that the whole protein in the liver of rats fed butter yellow is increased in the process of the liver cancer production, and, moreover, has ascertained that tyrosine is decreased throughout the entire process, while tryptophane is decreased at the begining and later increased in the liver during the experiment. In this paper the arginine contents of several tissues, that is liver, spleen, kidney, muscle aad brain of rats were estimated quantitatively in the process of liver cancer production by butter yellow feeding.

EXPERIMENTS

According to Kinoshita's method,[2] 50 albino rats were fed ad libitum on the mixture of polished rice (980 g) and cod liver oil solution of better yellow (20 g) so as to produce liver cancer. Green vegetables were given occasionally, and water too was supplied. 11 rats died early in the course of the experiment and they were discarded. At various periods in the course of the feeding, suitable number of rats were taden at random and the arginine values of the liver, spleen, kidney, muscle and brain were determined.

For the estimation of arginine the rat was narcotized with ether, and 0.5 g each of liver, spleen, kidney, muscle and brain tissues was weighed into a small morter and was throughly mashed with a small smount of emery. To the mashed tissue material was added 5 cc of distilled water and the whole was ground fine, to which was then added 10 cc of 20% hydrochloric acid and was hydrolysed. The hydrolysis was carried out in a boiling water bath for three hours. After the hydrolysis, each sample was neutralized with addition of 20% sodium hydroxyde and was then filtered. 0.5 cc of each filtrate was diluted with 4.5 cc of distilled water and was followed by the test for arginine (Sakaguchis' Reaction). The quantitative determination of the arginine was carried out with the colorimetric

* Aided by a Scientific Research Encouragement Grant from the Department of Education.

comparison by means of the Duboscq Colorimeter.[3)]

The standard color solution was prepared by the same reaction, of which concentration of arginine was 5 g per cc. The value of arginine content obtained was recalculated into mg per dl.

Up to the 75 day period, all the livers of rats were macroscopically normal with smooth surface. At the 100 day period, however, livers were no longer normal in appearance and the majority of them showed nodular hyperplasia or at least somewhat uneven surface. Over the 125 day period, all livers were cirrhotic. And at the 150 day period liver cancer with cirrhosis was to be seen very often.

The results obtained are presented in Table 1 in which the minimum and maximum mg dl of arginine are indicated and the mathematical average by the figures in brakets. It may be noted that the range of variation in the value of the various tissue may be often wide due to the individual variation. As may be apparent from the table, there was a marked increase of the arginine value of all the tissues, except one, and the increase reached quite a marked degree as early as at 25 to 50 day period. Besides the interesting feature of the early changes in the arginine value, the striking arginine increase was seen at the cirrhotic and cancer period. For instance the arginine value of the cirrhotic liver was $3/2$ times that of the normal liver.

The single exception was the spleen. There was a demonstrable reduction in the arginine value, which reached quite a marked degree at 50 to 75 day period.

It is noteworthy that the value of spleen arginine is reduced and that of other tissues is increased in the course of the liver cancer production.

Summary

To determine the arginine contents in liver, spleen, kidney, muscle and brain of the rats fed p-dimethylaminoazobenzene, Sakaguchi's reaction was followed at various days or stages during the experiment.

The striking fact was that the arginine values of liver and other tissues, except spleen, increased in proportion to the experimental days. Especially the peak of the arginine value in the liver was at the stage of cirrhotic liver.

On the other hand, the single exception was spleen, of which arginine decreased rapidly.

Table 1. The Average Value of the Arginine Content of the Tissues of Rats during the Course of Liver Cancer Production.

Feeding Days	No. of Rat	Liver	Spleen	Kidney	Muscle	Brain
0	10	360.0-431.1 (414.3)	556.5-652.2 (631.2)	407.1-441.9 (422.4)	410.1-504.0 (484.2)	367.2-445.5 (399.9)
(Controls) 10	5	451.5-535.8 (480.0)	528.0-753.6 (627.6)	457 8-496.2 (483.6)	402.0-596.4 (513.0)	424.2-496.2 (454.2)
25	6	447.9-550.8 (482.7)	457.8-668.4 (591.0)	564.0-744.0 (606.0)	495.9-656.4 (594.6)	437.4-566.1 (470.1)
50	6	441.9-561.9 (492.6)	488.7-718.2 (540.0)	496.8-603.9 (558.9)	522.0-694.2 (614.4)	447.0-522.0 (476.4)
75	5	462.0-522.6 (494.7)	457.5-621.0 (485.7)	532.5-607.8 (543.9)	495.6-574.5 (559.5)	430.5-558.6 (499.5)
100	5	469.2-573.9 (529.8)	454.5-517.8 (444.3)	496.5-561.0 (534.0)	543.0-589.5 (566.4)	522.6-567.0 (543.6)
Cirrhosis	7	501.0-714.0 (585.3)	418.2-471.0 (448.2)	561.0-687.6 (613.8)	544.5-597.0 (565.5)	522.6-624.3 (563.1)
Hepatoma	5	531.0-591.0 (555.0)	384.0-465.0 (443.4)	477.0-621.0 (543.0)	533.0-550.0 (547.0)	521.0-568.0 (552.0)

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要　旨

·肝癌生成過程に於ける白鼠臓器アルギニン

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p-Dimethylaminoazobenzene (Butter yellow) を木下法に従い白鼠に飼與して実験的肝癌を生成する過程における白鼠の肝・腎・脾・筋・脳等の臓器中のアルギニンを日を追うて定量した。臓器は 0.5 g 宛金剛砂で磨砕し，20% 塩酸 5 cc を乃至 10 cc 添加，湯浴上で 3 時間水解後，中和濾過し，濾液を 10 倍乃至 100 倍に薄め，坂口氏反應により 5 γ/cc 規準液と比色定量した。比色にはデュボスク比色計を用いた。

白鼠の肝所見に従って，肉眼的正常，表面不平滑，肝硬変並に肝癌の各期に分けて夫々の臓器アルギニンを比較したのであるが，肝・腎・筋・脳並に血清アルギニン含量は実験日数に従って増量し，肝硬変の際に最高値を示し，肝癌となって再び減量した。しかし正常値よりは遙かに増量している。

唯一の例外は脾アルギニン含量であって，実験開始後減量しはじめ，肝所見が表面不平滑を示す時期には正常値の $^{3}/_{2}$ を示す程度である。併し肝硬変乃至肝癌の時期には軽度の恢復を示す。

アルギニンは従來細胞分裂像の多い組織に多く含まれると報告されているが，所謂前癌状態と目される肝硬変の時期に肝臓のみならず他の臓器中にも最高含有量を示すことは，注目に値すると思う。尚脾臓が他の臓器とは反対にそのアルギニン量を減じる事は発癌機構の研究に何等かの示唆を與えるものではあるまいか。

(文部省科学研究費による)

[GANN, Vol. 42; March, 1951]

ON A BIOLOGICAL METHOD FOR THE DIAGNOSIS OF STOMACH CANCER. (FIRST REPORT)

RYUZO IWATSURU and ISAO KATO

(Internal Clinic, Wakayama Medical College)

In discussing early opinions on cancer of the stomach, Iwatsuru, in 1931, reached the idea that in the gastric juice of gastric cancer patients there should be some toxic substance, which might be demonstrable by way of certain changes in blood of laboratory animals. After various attempts, Iwatsuru and his co-workers (1937) found that after injecting stomach juice of stomach cancer patients intravenously to rabbits, an anemiogenic effect was testified, and published their experimental data in Folia Haematologica in 1937. They named this reaction provisionally "K. I. K. reation," and the anemiogenic factor "K. I. K. factor," taking the initials of Kozawa, Iwatsuru and Kawaguchi. They have studied then the technics concerning the K. I. K. reaction more closely, and tried it with a wonderful success for the diagnosis of stomach cancer. As their works have never been published except in Japanese (Kansaiiji, Shokakibyo, Gann, etc.) we will describe now the details of K. I. K. reaction.

Materials and Methods

The material to be used is the gastric juice taken from the patients suspected of gastric cancer. The gastric juice is to be taken early in the morning from the fasting stomach, using a Rehfuss tube or one of its modifications. The minimal necessary amount of it for a test is about 10 cc., but larger the amount used, the better result is obtainable. The pH of the gastric juice thus obtained is roughly estimated with test paper, and if it indicates acidity, neutralized with N/10 NaOH, then filtered through gauze to remove the mucus and food residues, if present. Thus we obtain an almost translucent fluid, which after dialysis with collodium membrane or cellophan paper against distilled water is kept in ice box to prevent putrefaction. The K. I. K. factor does not pass through the membrance. Sometimes a small amount of thymol is added when we keep it considerably long time (2 to 10 days or more.)

As experimental animals are used rabbits weighing 2 kg or thereabout. Empirically the sex of the animal has no relation to the reaction, but male is preferable. The animal is fed with fixed diet (such as bean curd refuse, rice bran, and vegetables) more than a week before the test and of course during the test. The blood specimen of the rabbit is to be taken from the ear vein and

its erythrocyte number is counted, and at the same time its hemoglobin estimation also is made, though the latter may be omitted.

After these procedures we take the test material out from the ice box, warm it, and inject it through the ear vein of the rabbit slowly. The amount of the gastric juice for injection is 1 to 2 cc. per kg. The injection is to be performed every day for 3 days successively and on the fourth day erythrocyte number and hemoglobin contents are again estimated, which are to be compared with the former values. To avoid error, we must use the same melangèur (diluting pipette), counting chamber and hemometer for the same animal before and after the injections. Moreover the estimation must be performed by the same person.

Interpretation of the results is as shown in the following table.

Reduction of erythrocyte number	over 31 %	(卌)
〃 〃	21——30 %	(卅)
〃 〃	11——20 %	(卄)
〃 〃	6——10 %	(+)
〃 〃	below 5 %	(0)
Increse of erythrocyte number		(−)

The positive reaction means gastric cancer.

The hemoglobin reading does not essentially serve for the interpretation.

To make sure, 2 rabbits are to be used at the same time, but this is by no means necessary. Only the careful estimation is indispensable.

Results

A. Healthy persons: 5 cases, all proved negative.

No.	Age	Sex	Gastric juice		Change in		Judgement of K. I. K. reaction
			free HCl	total acidity	Erythrocyte number (10^4) in/cmm.	Hemoglobin % (Sahli)	
1	38	♂	20	40	613→665	82→ 89	(−)
2	30	♂	10	20	540→650	90→102	(−)
3	35	♀	20	30	600→700	98→115	(−)
4	25	♂	15	24	550→630	91→100	(−)
5	18	♂	18	25	560→600	90→100	(−)

B. Various diseases except cancer (of the stomach): 24 cases, 21 of them proved negative.

No.	Age	Sex	Diagnosis	Gastric juice	K.I.K. reaction
1	40	♀	Gastric catarrh	anacid.	(−)
2	40	♂	Gastric catarrh	hypoacid.	(−)
3	35	♂	Achylia gastrica	anacid.	(−)
4	19	♂	Gastric ulcer	hyperacid.	(−)
5	54	♂	Gastric ulcer	anacid.	(−)
6	48	♂	Gastric ulcer	hyperacid.	(−)
7	57	♂	Gastroptosis	anacid.	(−)
8	63	♂	Pylorostenosis	normoacid.	(−)
9	64	♂	Pylorostenosis	anacid.	(−)
10	42	♀	Pylorospasm	anacid.	(−)
11	65	♂	Hepatocirrhosis	hyperacid.	(−)
12	26	♂	Jaundice	anacid.	(−)
13	50	♂	Cholecystitis	hyperacid.	(−)
14	55	♂	Cholelithiasis	anacid.	(−)
15	65	♂	Cardiac failure	hyperacid.	(−)
16	37	♂	Diabetes mellitus	hyperacid.	(−)
17	24	♂	Nephritis	hypoacid.	(−)
18	65	♂	Contracted kindey	hypoacid.	(−)
19	58	♂	Cancer of pancreas	anacid.	(−)
20	20	♂	Leukemia	anacid.	(+)
21	44	♂	Leukemia	normoacid.	(−)
22	36	♂	Werlhof's disease	anacid.	(−)
23	26	♂	Banti's disease	anacid.	(+)
24	58	♀	Simmonds' disease	anacid.	(+)

C. Gastric cancer cases: 56 cases, 54 of them proved positive.

No.	Age	Sex	Gastric juice	K.I.K. reaction	
				Erythrocyte count 10^4 in/cmm.	Judgement
1	50	♂	anacid.	570 → 406	(卅)
2	28	♂	anacid.	665 → 581	(卄)
3	40	♂	hyperacid.	693 → 520	(卅)
4	38	♀	hypoacid.	554 → 421	(卅)
5	47	♀	anacid.	440 → 380	(卄)
6	50	♀	anacid.	600 → 475	(卅)
7	42	♂	anacid.	521 → 437	(卄)
8	44	♀	normoacid.	465 → 432	(+)
9	32	♀	anacid.	611 → 442	(卅)
10	29	♂	anacid.	499 → 461	(+)
11	46	♂	anacid.	538 → 392	(卅)
12	51	♂	anacid.	644 → 552	(卄)
13	45	♂	hyperacid.	574 → 537	(+)
14	57	♂	anacid.	526 → 468	(卄)
15	55	♂	anacid.	532 → 484	(+)
16	48	♂	anacid.	615 → 551	(+)
17	58	♂	anacid.	588 → 514	(卄)
18	45	♂	anacid.	661 → 592	(+)
19	62	♂	anacid.	770 → 688	(卄)
20	61	♀	hyperacid.	820 → 760	(+)
21	56	♂	anacid.	568 → 551	(0)
22	44	♂	anacid.	574 → 615	(−)
23	54	♂	anacid.	559 → 452	(卄)
24	57	♂	anacid.	535 → 293	(卌)
25	43	♀	anacid.	553 → 490	(卄)
26	54	♂	hypoacid.	533 → 436	(卄)
27	61	♂	anacid.	493 → 372	(卅)
28	48	♂	anacid.	545 → 505	(+)

29	50	♀	anacid.	534 → 458	(+)
30	49	♀	normoacid.	548 → 504	(÷)
31	57	♂	anacid.	540 → 476	(+)
32	53	♀	anacid.	596 → 501	(+)
33	67	♂	anacid.	529 → 476	(÷)
34	63	♂	normoacid.	532 → 479	(÷)
35	68	♂	anacid.	468 → 408	(+)
36	58	♀	anacid.	631 → 555	(+)
37	53	♂	anacid.	747 → 611	(+)
38	50	♂	hypoacid.	556 → 505	(÷)
39	47	♀	anacid.	585 → 525	(÷)
40	53	♂	anacid.	594 → 521	(+)
41	60	♂	anacid.	650 → 580	(+)
42	52	♀	anacid.	474 → 415	(+)
43	52	♀	anacid.	474 → 431	(÷)
44	72	♂	anacid.	371 → 335	(÷)
45	50	♂	anacid.	485 → 396	(+)
46	59	♂	anacid.	423 → 391	(÷)
47	54	♂	hypoacid.	472 → 411	(+)
48	58	♂	anacid.	482 → 437	(÷)
49	41	♂	anacid.	464 → 402	(+)
50	65	♂	anacid.	505 → 415	(+)
51	50	♂	anacid.	421 → 379	(÷)
52	69	♂	anacid.	411 → 361	(+)
53	58	♂	anacid.	392 → 342	(÷)
54	52	♀	normoacid.	324 → 297	(+)
55	48	♂	hypoacid.	334 → 301	(÷)
56	52	♂	anacid.	388 → 333	(+)

Comment

After a large number of experiments, we are convinced of the practical value of the K. I. K. reaction. This method was tested by several other investigators with very satisfactory results. Over 90 % of the gastric cancer cases proved positive reaction, and Nakagawa and Oka reported 100% positive in their cases.

With regard to the stomack cancer diagnosis, there have been so numerous methods that we here withhold even to enumerate their names, but probably none of them ever succeeded with such a good results as ours. And moreover our method does not require any special apparatus or troublesome technics. So we believe that this method is very promising. We have found moreover that this anemiogenic factor in gastric juice of stomach cancer is something like protein or at least something closely combined to protein. Applying this fact we have succeeded to condense the factor much more, and developed a new method which will be described in the following report.

Conclusion

We described the details of our K. I. K. reaction, which is useful for the diagnosis of stomach cancer.

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要　　旨

胃癌診断の一生物学的方法に就て（第一報）

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著者等は胃癌胃液を家兎に靜脈内へ連続注射する時家兎赤血球数が著減する事実に著眼し之を胃癌診断に應用しようと努力して所謂 K. I. K. 反應を組み立てた。本篇にはその実施方法を精細に述べたものでその成績は極めて優秀である。

[GANN, vol. 42; March, 1951]

A BIOLOGICAL METHOD ON THE DIAGNOSIS OF STOMACH CANCER (SECOND REPORT)

RYUZO IWATSURU AND ISAO KATO
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In our first report, we described the original method of the K. I. K. reaction, a biological test for stomach cancer. Here, we will deal with its modification, a refined rapid method of the K. I. K. reaction.

As for the properties of the anemiogenic factor in the gastric juice of the gastric cancer patients, Iwatsuru and his co-workers had already found that this factor will not be destroyed by heating at 100°c for 5 minutes, and that it does not pass through the semi-permeable membrane.

Later, S. Nakagawa and his co-workers attained the same result by studying the character of the K. I. K. factor. They also added the fact that this K. I. K. factor is resistant not only against radium radiation (3414 mg hour), and exposure to ultra short wave (2.7 meter), but also against N/10 HCl, but nevertheless loses its activity by ultra violet ray (10 cm, 30 minutes) and by mixing with equal amount of N/10 NaOH. Noda and Tanaka also studied the same problem and arrived at a similar conclusion.

We further confirmed that this K. I. K. factor may be precipitated by heating at pH 5.0, or by adding sulfosalycilic acid or metaphosphoric acid in acid solution, and also by saturation with ammonium sulfate, and, moreover, that this factor is not soluble in ether.

These properties of the K. I. K. factor made us presume that it should be a sort of protein or at least something closely combined with protein.

We then applied our findings to condense the factor, and designed a modified method of the K. I. K. reaction. We prefered methyl alcohol to precipitate the factor after dialysation, and dissolved the resultant precipitate with a little amount of Ringer solution or distilled water which was injected hypodermically to a rabbit in order to test the anemiogenic effect.

Of course, the sensitivity of the reaction depends upon the amount of the anemiogenic factor which originates from the cancer tissue of the gastric wall. If the cancerous change in the stomach is in the early stage, and the amount of the factor from it minute, we may fail to demonstrate its presence.

For this reason, we contrived to condense the K. I. K. factor in order to sharpen the reaction, and consequently to make it useful for early diagnosis of gastric cancer. This new modified method, as we expected, proved to show

fairly good results. The details are given in the following section.

Methods

Gastric juice must be taken in the similar way as described in the first report. But this time, we collect as much as possible of the gastric juice, at least more than 20cc are desirable.

The gastric juice is then neutralized with N/10 NaOH in case of positive free HCl, and centrifuged to remove precipitate. We condense this upper fluid which is almost transparent, by means of evaporation at low temperature by using Faustheim's apparatus or simply with electric fan to reduce its volume to 1/3~1/10.

This procedure is not always necessary, because the intention of reducing the volume is only to save the methyl alcohol which is to be used in the next procedure. Then, a double volume of methyl alcohol for the fluid is to be added and centrifuged. This time we need the precipitate only, because the K. I. K. factor goes to the precipitate. If we add 2-3cc of distilled water to the precipitate and warm it a little while stirring, the precipitate dissolves easily. When it is necessary to remove methylalcohol, we must dialyse the solution more than 24 hours against distilled water.

Then we inject the solution (2 - 3cc.) to a rabbit weighing 2 - 3 kg hypodermically and count the erythrocytes number every hour after the injection; the number before the injection must be repeatedly checked.

In the estimation of the results we propose the following articles:

Increase of erythrocytes number	(−)
Decrease below 5 %	(0)
,, 6 ~ 9 %	(±)
,, over 10 %	(+)
,, over 20 %	(++)

The decrease of erythrocytes number in cases of gastric cancer usually occurs 1 - 2 hours after the injection, but in some cases may be delayed a little. Anyhow an observation within 4~6 hours is enough for the determination. We sometimes consult the value 24 hours after the injection as a reference.

Cautions which must be observed have been written in the first report.

Results

(1.) Cases of gastric cancer: all 9 cases proved positive.

No.	age	sex	Maximal decrease of erythrocytes number (10^4)	%	Judgement	Amount of gastric juice used (cc)
1	50	♂	89	21	(+)	35
2	52	♀	111	26	(+)	20
3	48	♂	58	11	(±)	25
4	52	♂	142	21	(+)	25
5	55	♂	63	12	(±)	40
6	28	♀	103	17	(±)	10
7	60	♂	135	24	(+)	30
8	51	♂	77	13	(±)	23
9	49	♀	107	17	(±)	18

(2.) Cases of stomach diseases which were obviously not cancer: they were 3 cases of ulcer (stomach or duodenum) 2 cases of perigastritis or peviduodenitis and 2 cases of gastric catarrh. All of them proved negative

No.	age	sex	Maximal decrease of erythocytes number (10^4)	%	Judgement	Diagnosis
1	33	♂	Increased		(−)	Gastric ulcer
2	32	♂	23	4	(0)	gastric ulcer
3	45	♂	19	3	(0)	duodenal ulcer
4	39	♀	19	4	(0)	perigastritis
5	36	♂	11	3	(0)	periduodenitis
6	56	♂	Increased		(−)	gastric catarrh
7	52	♂	Increased		(−)	gastric catarrh

(3.) Cases of various other diseases which were not cancer, including a few cases of healthy persons. All of them proved negative.

No.	age	sex	Maximal decrease of erythrocytes number (10^4)	%	Judgement	Diagnosis
1	36	♂	Increased		(−)	Kala-azar
2	32	♂	18	3	(0)	ankylostomiasis
3	54	♂	5	1	(0)	anacidity
4	65	♂	Increased		(−)	pulmonary tuberculosis
5	44	♂	Increased		(−)	constipation
6	58	♂	7	1	(0)	peritonitis
7	64	♂	Increased		(−)	ascariasis
8	32	♂	Increased		(−)	healthy
9	25	♂	Increased		(−)	healthy

SUMMARY

We have designed a modification of the K. I. K. reaction, which proved also considerably satisfactory results. We believe that our refined method is effetive for early diagnosis of gastric cancer, althouth we have not yet met with a suitable early stage of stomach cancer case to prove it.

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要　　旨

胃癌の一生物学的診断法に就て（第二報）

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著者等は胃癌胃液を家兎静脈内に注射して貧血を惹起せしむる，所謂ＫＩＫ反應を追試して之を確認し，第一報としてその方法を述べたが，更に胃癌胃液を透析した後，濃縮し，メタノールで沈澱せしめ，更に此の沈澱を生理的食塩水にとかして家兎皮下に注射する時，同様に催貧血作用を示すを確認した。それで之を胃癌の診断に用い得る様，方式を考按し，その成績を述べるものである。

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MODE OF ACTION OF TOXOHORMONE. A THIRD STUDY ON TOXOHORMONE, A CHARACTERISTIC TOXIC SUBSTANCE PRODUCED BY CANCER TISSUE

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INTRODUCTION

In 1948 we first isolated a thermostable, non-heat coagulable, water-soluble and alcohol-precipitable protein-like substance from various human tumors, both carcinomas and sarcomas, which has the property of markedly lowering the liver catalase of mice, and proposed for it the designation of "toxohormone" (1, 2). We later purified the substance to the point where it was active in 5 mg amounts, and concluded that toxohormone may be a kind of polypeptide (3). This is the toxic substance produced by malignant tissues which causes the characteristic lowering of the catalase activity of liver—the most striking of the systemic changes in cancer, repeatedly described by earlier authors (4—7) and actively investigated by more recent workers (8—14).

In a paper presented at the annual meeting of the American Association for Cancer Research, April, 1950, Greenfield and Meister (15) confirmed our original observations, using mouse mammary cancer and rat sarcomas, and also added considerable data as to the chemical properties of the "toxohormone fraction" they separated, which was active in 50—100 mg doses.

Pending the eventual isolation of toxohormone in a chemically pure state, which may be no easy task, we considered it profitable to turn our attention to the elucidation of the mode of its action, and the experiments to be reported in this paper bear directly upon this problem. We found that the toxohormone effect, namely. the lowering of liver catalase, can be nullified by the administration of excess iron to the animals.

In considering the mechanism of toxohormone effect on liver catalase activity, it is first of all important to point out that here no direct action, either destructive or functionally inhibitive, is involved, as has been fully demonstrated by the failure of tumor tissue (11) or toxohormone concentrate (2) to affect liver catalase *in vitro*. The only acceptable possibility which remains is that toxohormone may inhibit the synthesis of catalase. Our new finding that excess iron is capable of setting aside the toxohormone effect opens up an interesting vista regarding not only the mechanism of this inhibition of catalase synthesis, but also the

fundamental significance of toxohormone in cancer as related to the entire biological systems containing iron in the prosthetic group.

Experiments Based on Tumor Bearing Mice

Tumor used

In this series of experiments a transplantable sarcoma of mice discovered by us in 1948 and since maintained through serial subcutaneous transplantations in this laboratory was used. Histologically, the tumor is a spindle cell sarcoma with certain myxomatous features. It originated spontaneously in the subcutaneous tissue on the back of a female mouse of a strain kept by brother and sister mating for 5 generations. Several tumors, mostly mammary carcinomas, were found in other individuals of this strain of mice, but the sarcoma in question was the only one that proved to be transplantable to hybrid albino mice commonly used in this country. The rate of positive transplantation in these mice was not high at first, but after several generations its average transplantability became fixed at about 80 per cent. The growth is usually rapid, often producing tumors weighing 4—5 g in 3—4 weeks. Spontaneous regression sometimes occur among small tumors. Large subcutaneous tumors seldom ulcerate through the skin, though often invading the deeper tissues and penetrating through the peritoneum. They show the minimum of central necrosis and cystic or hemorrhagic changes are practically absent.

Unless otherwise stated all the mice were maintained on our usual laboratory diet of mixed grains or flour, supplemented with dried fish or fish meal and green vegetables.

Liver Catalase Activity in the Tumor Bearing Mice

It is first necessary to ascertain that the transplantable sarcoma on hand shows an adequate toxohormone effect, and for this purpose determinations of the liver catalase activity were made on several mice bearing the transplanted sarcoma.

For the determination of catalase activity we continued to follow the same simple, if not highly sensitive, gas-volumetric method, using a Battelli-Stern apparatus, which we adopted since our early experiments. The technical details of the method were exactly as described in our previous papers, and they need not be repeated here.

As may be expected, our transplantable sarcoma proved to be a good source of toxohormone, and, with the exception of a single case in which the tumor was of a small size, all those examined showed the typical, low activity of liver catalase, characteristic of all the tumor bearing animals so far investigated. The

data are presented below, together with those for normal control mice examined at the same time. It may be added that we have on hand a very extensive data on the liver catalase activity of normal mice, referred to in our previous papers, which show that no normal mouse gives the value of less than 4 cc oxygen, and that anything less than 5 cc is very rare.

Tumor mouse No.	Weight of tumor g	Liver catalase activity. Oxygen cc
1	5.2	1.7
2	4.7	2.1
3	4.2	4.2
4	3.5	2.1
5	3.2	4.5
6	2.9	2.3
7	1.5	5.0
8	1.4	3.4

Normal mouse No.		
1		10.5
2		8.1
3		8.0
4		6.5
5		6.4
6		6.0
7		5.5
8		5.2

Effect of Liver Feeding

In looking for possible methods which may serve as entering wedges into the problem of the mode of action of toxohormone, we decided first to search for a means whereby the toxohormone effect can be counteracted, believing that such an indirect approach more often yields an important clue than the direct attack.

It may be recalled that some years ago, while investigating the significance of liver catalase in the experimental production of liver cancer and its inhibition, we found that liver feeding not only inhibits the liver cancer production by an aminoazo dye but also prevents the lowering of the liver catalase activity, which invariably accompanies the process of carcinogenesis (16). It was this knowledge that suggested now the advisability of testing the effect of liver feeding.

Experiments were carried out using mice successfully transplanted with our sarcoma. When the tumor grafts became definitely established, the mice were transferred from the usual laboratory diet of mixed grains to the one supplemented

with liver. In order to facilitate handling, dried beef liver powder was added to mixed flour at the rate of 10 percent, and a sort of biscuit was made of the mixture. The mice were maintained on this liver-biscuit until their tumors grew to a sufficient size for our purpose, which was 10 days to 2 weeks. The age of the tumors at that time varied from 17 days to 3 weeks.

The liver catalase activity in these tumor bearing mice, maintained on the liver-supplemented diet, was determined in the same way as before, and the results left no doubt that liver feeding adequately prevented the lowering of liver catalase, so characteristic of tumor bearing animals. In normal, non-tumor bearing mice, the effect of liver feeding was negligible, although the liver catalase activity in these mice tended to be slightly higher on an average than in non-liver fed controls.

Tumor mouse No.	Duration of liver feeding. Days	Weight of tumor g	Liver catalase activity Oxygen cc
1	10	2.7	6.5
2	〃	2.6	8.1
3	〃	2.4	8.4
4	14	3.2	6.3
5	〃	3.1	7.1
6	〃	2.8	4.1
7	〃	2.6	5.8
8	〃	2.5	4.8
Normal mouse No.			
1	14		10.5
2	〃		9.1
3	〃		8.6
4	〃		7.5
5	〃		7.0
6	〃		6.4
7	〃		5.6

Effect of Blood Meal Feeding

The positive effect of liver feeding above described led us to test the effect of the other material which our previous investigations showed to likewise inhibit the liver cancer production by an aminoazo dye and to prevent the accompanying lowering of the liver catalase activity, namely, blood meal (17).

Blood meal was prepared by simply evaporating to dryness and pulverizing the "blood cake" left over after the removal of serum from defibrinated horse blood. It was added to mixed flour at the rate of 10 percent and was made into

biscuit. As in the preceding experiment, mice with small but definitely established sarcoma transplants were placed on this diet for 10 days to 2 weeks, at the end of which period the mice with the tumors of sufficient size were killed and their liver catalase activity determined.

As may be seen in the table below, all the mice, with the exception of one with an extra large tumor, showed approximately the normal liver catalase activity:

Tumor mouse No.	Duration of blood feeding. Days	Weight of tumor g	Liver catalase activity Oxygen cc
1	10	6.5	2.9
2	〃	3.6	7.0
3	〃	3.1	5.0
4	14	3.2	5.7
5	〃	3.1	4.7
6	〃	3.0	5.9

Effect of Iron Feeding

Our speculation based on the results of the two preceding experiments suggested that the active agent in liver and blood which counteracted the toxohormone effect might possibly be iron (cf. 18) In this third experiment, therefore, an attempt was made to determine whether or not iron feeding would also prevent the lowering of liver catalase activity in tumor bearing mice.

Ferric chloride ($FeCl_3.6H_2O$) was added to mixed flour at the rate of 5 mg per 300 g and biscuit was made as in the foregoing experiments. If we assume the daily consumption of the biscuit by a mouse to be 3 g this amount of ferric chloride would come to 0.05 mg per mouse per day, which, on body weight basis, corresponds to nearly fifty times the amount generally held to be the normal, physiological requirement.

As before, mice with established but small sarcoma transplants were fed on this iron diet for 10 days to 2 weeks, and the tumors were allowed to grow to suitable sizes. The catalase activity of liver of these mice was determined at the end of this period.

To our satisfaction, the result of this experiment agreed well with those of the preceding two, and demonstrated that iron in the simple form of $FeCl_3.6H_2O$ was quite adequate in nullifying the toxohormone effect.

Tumor mouse No.	Duration of Iron feeding. Days	Weight of tumor g	Liver catalase activity Oxygen cc
1	10	8.2	3.7
2	〃	3.7	5.8

3	〃	3.2	7.0
4	〃	2.9	6.2
5	〃	2.0	5.7
6	14	4.6	4.6
7	〃	3.9	4.8
8	〃	3.4	4.8
9	〃	3.0	5.6
10	〃	2.6	6.0

Catalase Activity of Sarcoma Tissue

Incidental to making the liver catalase determinations on the sarcoma bearing mice in the preceding experiments, opportunity offerred itself of studying the catalase activity of the sarcoma tissue under various dietary conditions.

The catalase activity was measured exactly in the same way as in the case of liver catalase, except that a larger amount, 0.5 g, of sarcoma tissue was used. Active layer of each tumor was chosen, carefully avoiding any necrotic area and peritumoral normal tissues.

It is well known that tumor tissues in general are deficient in catalase, and the mouse sarcoma we used proved to be no exception to this rule. The point which may be of interest in the results obtained is the occurrence of exceptionally high figures among the iron fed group (marked with * in the table below), but the data are insufficient to establish the significance of these variations. Pending the elucidation by further experiments we simply record the findings here for what they may be worth, without comment.

Tumor No.	Catalase activity (Oxygen cc) under various diets			
	Without Supplement	With liver	With blood	With $FeCl_3$
1	0.75	0.56	0.60	*2.40
2	0.27	0.46	0.38	*2.30
3	0.28	0.29	0.28	*1.70
4	0.18	0.28	0.28	*1.60
5	0.05	0.27	0.19	*1.40
6			0.18	*1.40
7				1.08
8				0.65
9				0.38
10				0.28
11				0.18
12				0.18
13				0.06

Experiments Based on Injections of Isolated Toxohormone

Having established the fact that iron (or iron-rich materials) counteracts the toxohormone effect in tumor bearing animals, our next task was to determine whether or not the liver catalase lowering effect of the injections of isolated toxohormone can also be set aside by iron.

Isolation of Toxohormone from Mouse Sarcoma and Its Effect on Iron-fed Mice

Fresh tissue of transplanted mouse sarcoma collected from a large number of mice was dried in an open dish over boiling water bath. 50 g of dried sarcoma tissue was pulverized and extracted with 1 *l* of distilled water, well stirring and heating for 1 hour. Watery extract was evaporated to about 100 cc over boiling water bath, and after centrifuging off the coagula which formed during the evaporation, two volumes of absolute alcohol was added and mixed. The precipitate formed by alcohol was dissolved in distilled water and $CuSO_4$ solution was added so as to make the $CuSO_4$ concentration just 1 percent. The bluish precipitate formed was washed with distilled water, and then treated with N/10 HCl solution to remove copper. The final pure white precipitate was washed with 70 percent alcohol, dried, and washed with ether. Yield: 700 mg.

The above procedure is exactly the same as we previously described as a standard method for the purification of toxohormone from human tumors (3), the only deviation consisting in the washing of copper-removed precipitate with 70 percent alcohol, instead of with distilled water, which latter being apt to reduce the yield.

The activity of the toxohormone isolated from mouse sarcoma as above was tested by injecting into normal mice and determining the liver catalase activity 22 hours after the injection. It proved to be active in 10 mg but not in 5 mg doses.

Mouse No.	Amount injected. mg	Liver catalase activity (Oxygen cc)
1	5	5.8
2	〃	5.3
3	10	4.4
4	〃	4.3
5	〃	4.5
6	〃	4.3
7	20	4.3
8	〃	4.3
9	〃	4.0
10	〃	3.6
11	〃	3.6

This sample of toxohormone saw tested on mice maintained on the biscuit containing 5 mg $FeCl_3 \cdot 6H_2O$ per 300.g. The duration of the iron feeding before the injection was 2 weeks, and the liver catalase determination was made 22 hours after the toxohormone injection.

The result, as tabulated below, clearly demonstrated that iron feeding adequately counteracted the liver catalase lowering effect of toxohormone.

Iron.fed mouse No.	Amount of toxohormone injected. mg	Liver catalase activity Oxygen cc
1	10	8.5
2	〃	7.0
3	20	6.8
4	〃	6.6
5	〃	5.5
6	〃	5.0
7	〃	4.5
8	〃	2.6

Effect of Toxohormone Isolated from Human Tumors on Iron fed Mice

In order to confirm the above result, experiment was repeated using a pooled sample of toxohormone isolated as coppor salt from various human malignant tumors (3). Although the sample was over one year old since preparation it had lost none of its activity. Its effect, however, was definitely inhibited in iron fed mice, except in the cases of a large dose of toxohormone.

Iron fed mouse No.	Amount of toxohormone injected. mg	Liver catalase activity Oxygen cc
1	25	4.6
2	〃	4.1
3	〃	3.9
4	7.5	9.0
5	〃	7.0
6	〃	6.4
7	〃	6.0
8	5	6.6
9	〃	6.6
10	〃	6.5
Non-iron fed control mouse No.		
1	25	3.5
2	7.5	4.3
3	〃	4.0
4	5	4.4
5	〃	4.2

The apparent quantitative relation existing between the available iron and toxohormone injected may be pointed out as of special interest.

Effect of Injecting Ferric Chloride mixed with Toxohormone

In this last experiment of the series attempt was made to see if iron administered simultaneously with toxohormone would counteract the effect of the latter. For this purpose the same samples as used before of toxohormone isolated from mouse as well as from human tumors were dissolved in distilled water, and were mixed with solutions of $FeCl_3 \cdot 6H_2O$, both solutions being steam-sterilized beforehand, and the mixtures immediately injected intraperitoneally into normal mice in appropriate amounts. The catalase determinations were made 22 hours after the injection.

From the results obtained, which are tabulated below, it seems certain that iron injected simultaneously is capable of nullifying the effect of toxohormone. A question may be raised as to the possibility of $FeCl_3$ inactivating toxohormone in the test tube, but the occurrence of a few exceptional cases in which the effect of iron was not shown (marked with * in the table) may be taken to speak against this idea.

Material injected	Mouse No.	Liver catalase activity. Oxygen cc
Mouse toxohormone 20 mg +$FeCl_3$ 15 mg	1	8.2
	2	7.2
	3	7.2
	4	7.0
	5	6.4
	6	5.9
Mouse toxohormone only 20 mg	1	4.3
	2	4.3
	3	4.0
	4	3.6
	5	3.5
Human toxohormone 7.5 mg +$FeCl_3$ 1.25 mg	1	7.2
	2	6.3
	3	5.8
	4	5.3
	5	*4.9
	6	*4.8
	7	*3.8
Human toxohormone only 7.5 mg	1	4.3
	2	4.0

Chart 1. Effect of diets on liver catalase activity of tumor bearing mice.

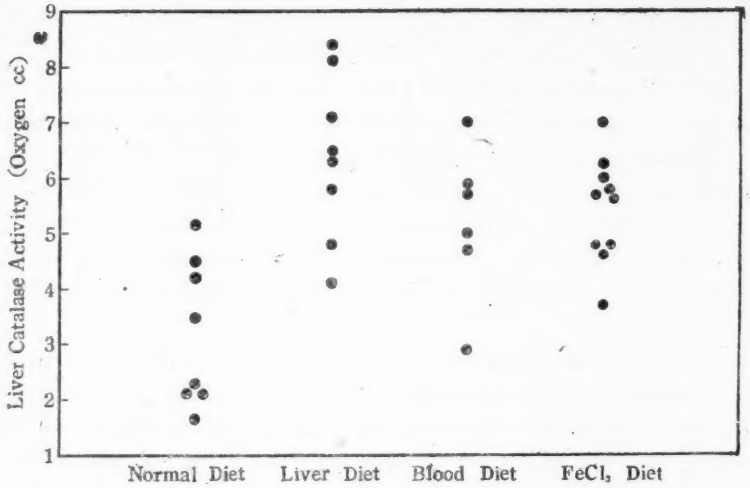
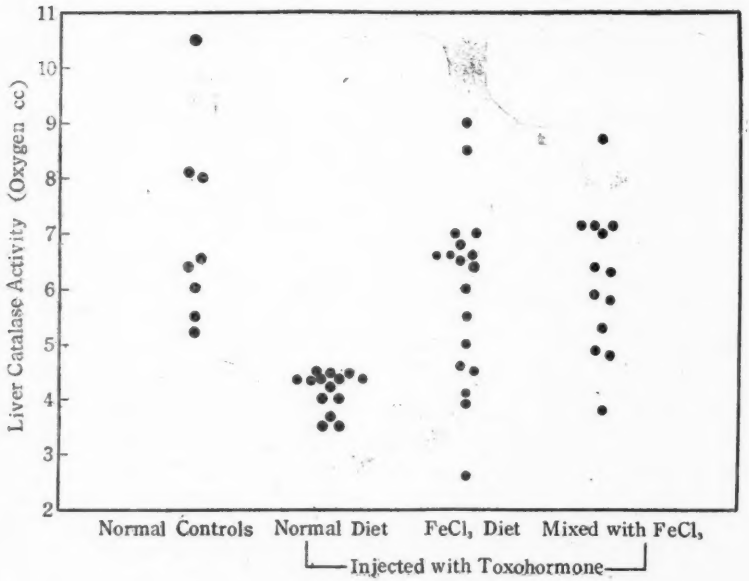


Chart 2. Influence of iron on the effect of injections of toxohormone.



DISCUSSION

Even before the discovery of toxohormone, there has been a strong presumption that the marked lowering of the liver catalase activity in the presence of

growing malignant tumor is due to the interferance of the synthesis of catalase in the liver (19). The complete failure to find any effect whatever on catalase *in vitro* of toxohormone or other tumor materials excludes the possibility of direct inhibition. Greenstein's observation (11) that on dialysis there is the same proportionate drop in activity for extracts of normal livers and of the livers of tumor bearing animals proves the absence of dissociable inhibitor, and the fact that the kinetic behavior of catalase is identical in both kinds of liver adds another evidence for the same conclusion. There seems to be no doubt that the low liver catalase activity in tumor bearing animals is ascribable to the acutal low catalase content, which can occur through the reduced rate of catalase production in the liver.

Experiments reported in this paper demonstrated that a simple iron compound in the form of $FeCl_3 \cdot 6H_2O$, when supplied in a sufficient amount either in food or by injection, adequately prevents the lowering of liver catalase, which otherwise occurs constantly in tumor bearing animals or in normal animals injected with toxohormone concentrate. The toxohormone effect must be closely connected with the deficiency of iron, since the extra supply of iron can nullify the effect. It may safely be assumed that it is not the ability of liver cells to synthesize catalase that is damaged, but rather the shortage of necessary iron that is caused by toxohormone.

From the point of view that toxohormone inhibits the synthesis of catalase, our new evidence permits the inference that toxohormone may be a strongly siderophilic substance, binding iron so fast as to markedly reduce the amount of available iron for the catalase synthesis. Catalase is a sort of conjugated protein with hematoporphyrin prosthetic group.

In order to establish this hypothesis, however, it is necessary to demonstrate the formation of toxohormone-iron complex by isolating it. It is known that siderophilin, a plasma protein, forms with inorganic iron a stable, salmon pink complex. Aspergillic acid and hydroxylamine are also known under certain conditions to produce a similar complex with iron (20). If our hypothesis is correct it may be possible to show in the future that toxohormone combines with iron to form a fairly stable complex.

The fundamental concept we have developed that toxohormone may act through its iron-binding property brings us face to face with a problem of much wider significance than hitherto suspected, since not only catalase but also all other iron-containing systems may now be visualized as being possibly affected by toxohormone. Hemoglobin, cytochromes and cytochrome oxidase all come under this category, and it may be that the synthesis of these important substances is interfered with by toxohormone, depending on the amount of the latter pro-

duced by given tumors.

Another aspect of the toxohormone effect, hitherto totally neglected, is the possible relation to the characteristic metabolic pattern of cancer cells, since the constant presence of toxohormone may well have something to do with the habitually low iron-containing enzyme systems in these cells.

There is much to be learned as to the significance of toxohormone in cell physiology of cancer and of cancerous animals.

Summary

The toxohormone effect, that is, the lowering of liver catalase in tumor bearing animals, can be counteracted by dietary supply not only of dried liver or blood powder but also of excess ferric chloride. This fact was demonstrated in the case of mice bearing transplanted sarcoma and also of normal mice injected with potent toxohormone isolated from human as well as mouse tumors. Injections of ferric chloride mixed with toxohormone in solution also prevented the lowering of liver catalase, which toxohormone alone would bring about.

Based on these experimental results a hypothesis was advanced that it may be through its property of binding available iron that toxohormone interferes with the normal synthesis of catalase, and the possible significance of toxohormone in relation to the whole of iron-containing enzyme systems in cancer was discussed.

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要　旨

トキソホルモンの作用機轉

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1948 年我々は初めて人の悪性腫瘍よりマウスの肝臓カタラーゼ作用を，顯著に減少せしめる物質卽ち，化学的には耐熱性で，熱に凝固せず，エーテルに不溶，水によく溶け，アルコールに沈澱する物質を分離し，之をトキソホルモンとなづけた。

この仕事は 1950 年 Greenfield 及 Meister により動物腫瘍を用いて追証されている。我々は更にこれを銅塩で沈澱せしめて精製して，この物質はポリペプチードであろうと推定しているが，之を化学的純物質として取り出す事は極めて困難であると思われる。

本文にはトキソホルモンの作用機序に関する新知見を報告した。

このカタラーゼの減弱は飼料に肝粉，血粉或は塩化第二鉄を過剩に添加する事により防止しうる。この事実は移植腫瘍を持つマウス，健康マウスにトキソホルモンを注射した場合はもとより，トキソホルモンと塩化第二鉄を混じて注射した場合にもいづれも同じく証明された。

トキソホルモンの作用機序を考察するに，直接カタラーゼを破壞或はその作用を阻害するものではなく，合成の阻害である事はすでに推定されているが，我々の新知見において，トキソホルモンは鉄と優先的に結合する事によつて，カタラーゼの合成を阻害するのではないかという考えに到達する。この考えが正しければ，鉄を作用基とする他の酵素系もトキソホルモンの影響を受けるのではないかという問題を提供する事になる。

文部省科学研究費による。

[GANN, Vol. 42; March, 1951]

ON THE LIVER-ENZYMES OF RATS FED WITH P-DIMETHYL-AMINOAZOBENZENE, ESTERASE AND CATHEPSIN

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PART I. ESTERASE

Several years ago one of the authors[2] found that esterase activity of a transplanted hepatoma (Iikubo strain) is markedly lower than that of a normal liver and so low as 1/3—1/4 of the latter. Greenstein et al.[1] pointed out in the reports of their comprehensive study on enzyme activity of homologous rat liver tissue, that the esterase activity of normal and regenerating liver is evidently higher than either transplanted or primary hepatoma. It has attracted our attention that enzyme activity of a fetal liver was rather close to that of a neoplastic liver.

Subsequently we came to have an idea that it may afford some solution to the question of malignancy if we study enzymologically the course of experimental liver cancer production in rats fed with p-dimethylaminoazobenzene (DAB) under classification regarding pathological changes of the liver instead of according to the duration of feeding.

In this case we should prove beforehand that DAB itself does not affect enzyme activity in vitro. Furthermore we added as a control, livers of rats which have been fed more than forty days on polished rice supplemented with beef liver powder. According to the finding of Nakahara et al.[5] the liver feeding has a remarkable effect in inhibiting the occurrence of liver cancer in rats fed with azo dyestuffs.

MATERIALS

After feeding with DAB more than 100 days by the well known method of Kinoshita, rats were killed and their livers were classified macroscopically according to the suggestion of Nakahara et al.[6] into following four groups, namely, macroscopically normal liver (I), liver with uneven surface (II), cirrhotic liver (III) and hepatoma (V). One more group was added (5 groups in total), that is, the non-cancerous portion of cirrhotic liver with a nodular hepatoma (IV), which was considered to be worthy of study biochemically.

For the control groups liver of normal rat (2) fed solely polishee rice and that

of rats fed with beef liver diet (1) were provided. In each group 2—10 experimental animals were used.

Methods

Enzyme solution, glycerol extract of tissue, was prepared as follows: to the paste of 3 g (wet weight) of tissue glycerol was added, stired thoroughly and its total volume was filld up just to 10 cc by the latter.

To 0.5 cc of this solution (corresponds to 0.15 g of original tissue) 6 cc of distilled water (previously adjusted to pH 7 with NaOH) and 0.5 cc of substrate diluted with 3 cc of glycerol were added. After addition of a few drops of toluene the flask was stoppered tightly and incubated at 37°C for 24 hours. Whole mixture was titrated with 0.1 N NaOH against phenolphthalein and the titration value of the blank tests (similary made up solution mixture but without substrate) was subtracted from it.

Ciphers in Table 1 and figures represent 2/3 of the value obtained by titration. Thence they indicate how much 0.1 N NaOH was required to neutralize acid produced by hydrolysis, when 0.1 g (wet weight) tissue is incubated at 37°C with substrate for 24 hours.

As substrate, tributyrin (Merck), triacetin (b. p. 258—260°), methylacetate (b. p. 54—56°) and ethylpropionate (b. p. 97—99°) were chosen.

The experiments in which DAB was added to tissue extract in vitro were made as follows: 0.5 cc of enzyme solution, 0.5 cc of tributyrin, 3.0 (3.5) cc of glycerol, 1.0 (0.5) cc of 0.2 per cent solution of DAB, containing 2 (1) mg of DAB crystal, and 5 cc distilled water were mixed thoroughly and incubated at 37°C for 24 hours. Titration value multiplied by 2/3 are presented in Table 2.

Table 1.

Substrate	Control		DAB Fed				
	Liver Fed	Normal Diet	Macroscopically Normal	Uneven Surface	Cirrhotic Liver		Hepatoma
	(1)	(2)	(I)	(II)	(III)	(IV)	(V)
Triacetin	5.01	3.99	3.88	3.47	3.36	3.61	1.85
Tributyrin	2.52	2.06	2.16	1.81	1.52	1.77	0.96
Methylacetate	3.04	3.12	3.17	1.93	1.89	1.92	0.67
Ethylpropionate	5.41	4.45	4.71	3.91	4.10	4.31	1.76

Table 2.

Substrate: Tributyrin

Enzyme Solution	No. of Exp.	DAB mg per 0.1 g Tissue	cc 0.1 N NaOH
Normal Rat Liver	1	—	2.48
		0.66	2.76
		1.33	2.72
	2	—	2.37
		0.66	2.21
Hepatoma	3	—	1.45
		0.66	1.54

Summary and Comments

All data were summarized in Table 1 and represented graphically in Figures 1-4.

It was substantiated that esterase activity of the liver was lowered just at the stage when the pathological change was first recognized macroscopically in the liver and was not related to what substrate was used. In the stage of hepatoma its enzyme activity was lowered markedly. Esterase of non-neoplastic part of a liver containing hepatoma nodules had a little higher activity than in liver which has suffered from general cirrhotic change.

Among control groups it is noticeable that the esterase activity of the liver fed rats was higher than that of normal rats. But the inhibitory action of liver feeding on hepatic cancer production may have no correlation to the above fact.

There was no difference between esterase activities of glycerol extract of liver whether DAB was added to it or not in vitro. In these experiments quantitative ratio of added DAB to the amount of tissue was about 1000 times larger than that which was determined by Masayama et al.[4] in the liver of DAB fed rat. In glycerol extract of hepatoma no difference was also found in vitro whether DAB was added or not.

Part II. Cathepsin

One of the authers previously compared cathepsin activity in normal rat liver and transplanted hepatoma (Iikubo strain) and found that of the latter is lower than of the former.[3]

Material and Methods

Materials were prepared similary as in experiments of esterase.

I

Enzyme solution: glycerol extract of tissue, 1 cc of which corresponds to 0.3 g

wet weight of the original tissue, 1 cc, and buffer solution of pH 5 (1/5 M citrate mixture), 3 cc. Substrate: 2 per cent Hammarsten casein (Merck) solution, 5 cc, and distilled water, 1 cc. Whole mixture, filled up to 10 cc, and added with a few drops of toluene, was incubated at 37°C for 24 hours. Formol titration of the mixture was made using 5 cc of the mixture. After adding 5 cc of 3 times diluted formalin which has been made slightly alkaline against phenolphthalein titration was made with 0.1 N NaOH to obtain the required cc number. The remaining 5 cc was kept for further 48 (in total 72) hours in the incubator and titrated.

Activation experiment by cysteine was carried on similary as above. Cysteine-hydrochloride was added to the concentration of 0.1 M to 1 cc of tissue glycerol extract and adjusted to pH 5 shortly before mixing. 30 minutes later 5 cc of substrate and 3 cc citrate buffer were added and treated similary as described above. From all cc numbers those of blank test which was determined in solution mixture whithout substrate, were subtracted.

In Table 3 and Figures 5—6 1/3 of obtained cc numbers are presented, thence they indicate how many cc of 0.1 N NaOH were used to neutralize prodbced amino acid by the enzyme in 0.1 g tissue, when it was incubataed at 37°C for 24 or 72 hours with 1 per cent casein.

Experiments with liver extract of normal rats to which DAB was added in vitro, were carried on as follows: substrate (5 cc), buffer solution (3 cc), tissue extract (1 cc) and glycerol solution of DAB with distilled water (1 cc) were stired and incubated at 37°C.

Ciphers in Table 4 represent the 1/3 of the cc used for titration, from which numbers of blank test have previously been subtracted. Thence they indicate required cc of 0.1 N NaOH in formol titration when extract of 0.1 g (wet weight) of tissue digested 1 per cent casein at 37°C under addition of DAB in vitro.

Table 3

Substrate	Hours	Control		DAB Fed				
		Liver Fed	Normal Diet	Macroscopically Normal	Uneven Surface	Cirrhotic Liver		Hepatoma
		(1)	(2)	(I)	(II)	(III)	(IV)	(V)
Casein 1 %	24	0.259	0.179	0.349	0.259	0.346	0.129	0.069
	72	0.392	0.339	0.556	0.489	0.432	0.456	0.266
Casein 1 % +Cysteine	24	0.289	0.253	0.286	0.296	0.336	0.273	0.159
	72	0.572	0.512	0.549	0.619	0.626	0.512	0.479

Table 4

Substrate: Casein 1 per cent.

Enzyme Solution	No. of Exp.	DAB mg per 0.1g Tissue	cc 0.1 N NaOH	
			24 hrs.	72 hrs.
Normal Rat Liver	1	—	0.268	—
		0.66	0.250	—
	2	—	0.280	0.293
		0.33	0.213	0·293

Summary and Comments

Whole data were summarized in Table 3 and Figures 5—6. Cathepsin activity of liver increased by DAB feeding exceeding that of normal one and was lowered markedly when hepatoma developed. In the latter case the activity was much lower than the normal. Though the cathepsin activation by cysteine was seen both in normal liver and hepatoma, it was not so distinguishable in liver of rat fed with DAB (except hepatoma), that is, in the macroscopically normal liver or cirrhotic liver. It was thought that these facts might be accounted for if we assume that cathepsin has been already activated in all these cases.

Differing from experiments with esterase, that the occurrence of larger activation than control was already distinguishable even in the stage of macroscopically normal liver, may afford some information on the study of precancerous stage.

Among control experiments activity of cathepsin in the liver of beef liver fed rat was a little higher than that of rats fed with ordinary diet, and in the experiments of activation by cysteine the similar difference was estimated.

Furthermore, as shown in Table 4, there was found no discrepancy of cathepsin activity between original enzyme solution from normal rat liver and the solution added with DAB in vitro.

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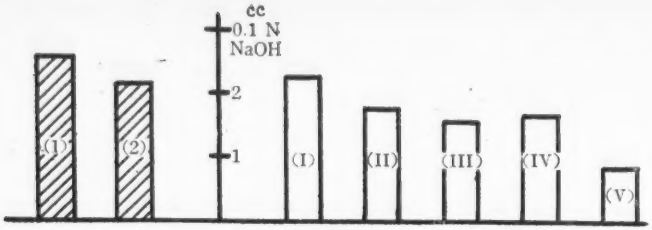

Fig. 2.
Sub : Tributyrin

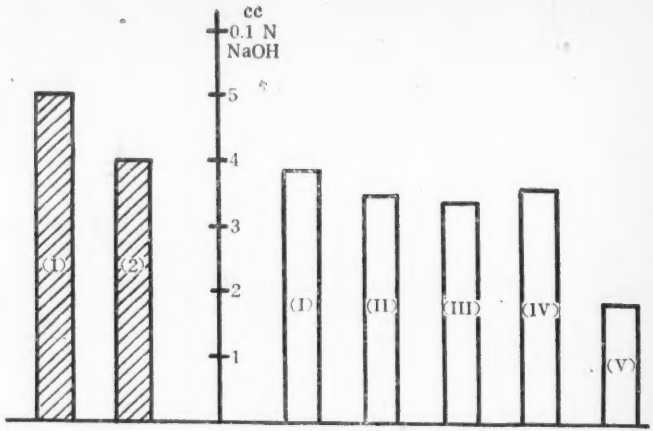

Fig. 1.
Sub : Triacetin

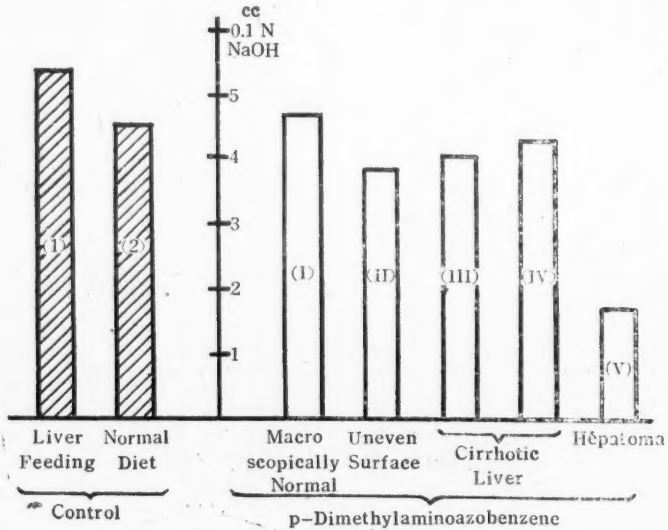

Fig. 4.
Sub : Ethylpropinate

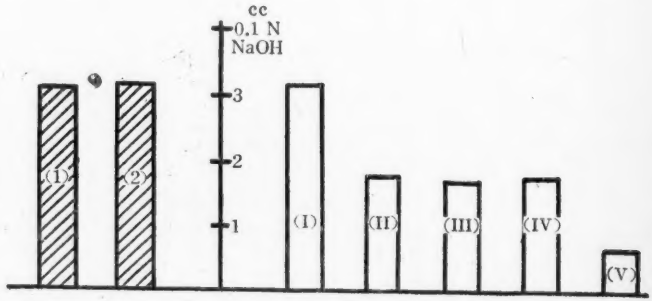

Fig. 3.
Sub : Methylacetate

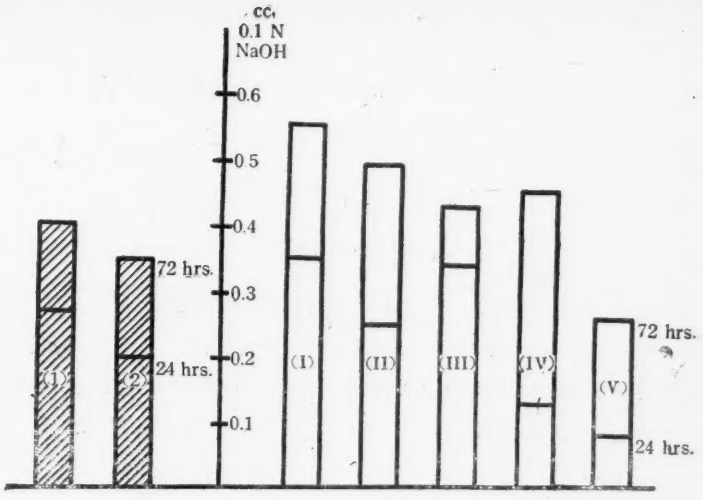

Fig. 5.
Sub: Casein 1 per cent

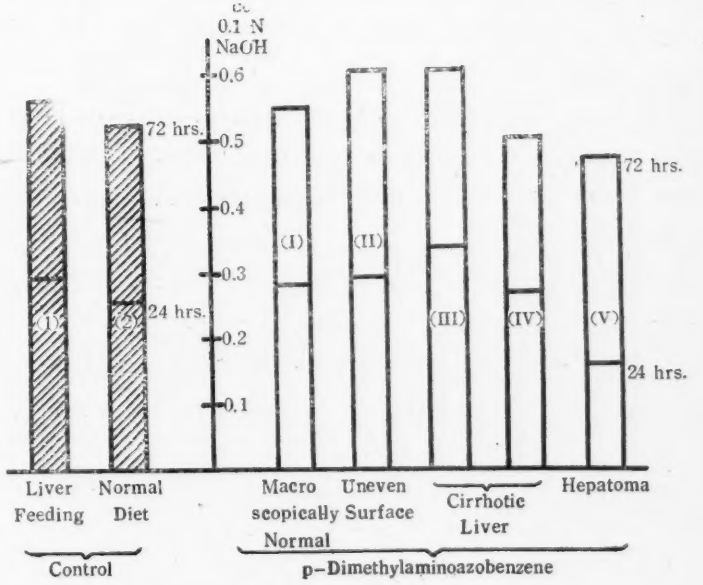

Fig. 6.
Sub: Casein 1 per cent. +Cysteine

要　旨

実驗的肝癌生成過程に於ける肝酵素に就て
エステラーゼ及カテプシン

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バターイエロー投與100日以上經過した白鼠の肝の病変度を肉眼的に觀察して区分し，夫々について酵素作用を調べた。その区分は肉眼的正常，表面不平滑，硬変及び肝癌で，尚これに肝癌生成肝の非癌部の硬変を加えた。対照に普通食（白米）の白鼠の肝と肝癌生成阻止物質である牛肝投與白鼠の肝を採った。

酵素液（グリセリン抽出液）と基質を混じ一定時間孵卵器中に放置し，エステラーゼの場合は中和するに要する 0.1 N の NaOH の cc 数で活性度を表し，カテプシンの場合はフォルモール滴定により，その活性度を 0.1 N の NaOH の cc 数で示した。

エステラーゼ作用はバターイエロー投與により肉眼的変化が表われる時期に作用が下り，硬変期は同様な値を示し，肝癌は更に顯著に低下している事が認められた。尚対照の内牛肝投與のものは普通食のものよりエステラーゼ作用の大なる事が認められるが，この事実と肝癌阻止作用との関連性は不明である。

カテプシン作用はバターイエロー投與により正常より增し肝癌生成されるに及んで顯著に活性を減ずる，その時は正常よりも更に低い，カテプシンのチステインに依る賦活作用は正常肝と肝癌に認められるが，バターイエロー投與動物の肝（肝癌を除く）の肉眼的変化のない肝や硬変の肝には明らかでない。

対照にとった正常のうち牛肝投與と普通食の白鼠肝のカテプシン作用は前者が稍大である。賦活後にもその差異は認められる。

尚正常肝の酵素液に in vitro でバターイエローを添加した実驗ではエステラーゼ及びカテプシンともに元の酵素液と作用の差異は認められなかった。　[文部省科学研究費による]

[GANN, Vol. 42; March, 1951]

ON THE SULFER CONTENT OF THE LIVER PROTEIN OF RATS FED WITH P-DIMETHYLAMINOAZOBENZENE

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INTRODUCTION

Apparently it is highly significant to compare sulfer content of protein in neoplastic and normal tissues, as S-S linkage in protein is considered to be one of those which play a rōle to maintain the structure of protein molecules. Greenstein and Leuthardt [1] studied on sulfer distribution by determining sulfer containing amino acids in water extractable protein of tissue and pointed out a close resemblance of sulfer distribution between hepatoma and fetal livers.

Attempts were made in this study to investigate ebbs and flows of sulfer content of protein in the course of liver cancer production in rats fed with p-dimethylaminoazobenzene (DAB).

MATERIAL

The materials were quite similar to those which have been used in our study on esterase and cathepsin.[2]

Rats were killed after feeding with DAB more than 100 days. Livers were taken out and classified in the following 5 groups based on their macroscopical pathological changes: macroscopically normal liver (I), liver with uneven surface (II), entirely cirrhotic liver (III), noncancerous (cirrhotic) portion of liver which includes hepatoma nodules (IV) and hepatoma (V). As control groups livers removed from rats fed with beef liver for 40 days (1) and those fed with ordinary diet (polished rice) (2) were used. In each group 2-5 rats were provided.

METHODS

Excised livers were finely cut by scissors till it became paste like consistency and dried in a desiccator. After being ground to powder and treated with hot ethanol to remove fatty substances thoroughly, the material was again preserved in a desiccator. For quantitative analysis of sulfer Liebig-Du Menil's method was employed. Nitrogen content was determined by micro Kjeldahl method. Details of these methods were summarized in another report published by one of the authors (S. Kishi) and his coworkers.[3]

SUMMARY AND COMMENTS

In Table 1 average S-N ratio in every group of experimental animals was

presented. The S-N ratio was calculated from the result of sulfer and nitrogen determinations in the powdered liver. Therefore maximal and minimal values of S/N do not coincide strictly with those which are calculated from S and N values shown in respective columns of the table, as S/N is determined in every case independently. In Fig. 1 average S mg/N mg×10 of each group was represented graphically and in Fig. 2 mean values of N mg/10 mg protein were shown.

Table 1.

		Control		p-Dimethylaminoazobenzene Fed				
		Liver Fed	Normal Diet	Macroscopically Normal	Uneven Surface	Cirrhotic Liver		Hepatoma
		(1)	(2)	(I)	(II)	(III)	(IV)	(V)
No. of Exp.		5	3	5	4	4	2	2
S mg / 100mg Protein	Max.	1.316	1.342	1.15	1.082	1.137	1.562	1.398
	Min.	0.178	0.972	0.411	0.849	0.74	0.905	1.342
	Mean	0.638	1.168	0.824	1.013	0.955	1.233	1.370
N mg / 10 mg Protein	Max.	1.473	1.430	1.587	1.528	1.541	1.507	1.549
	Min.	1.223	1.318	1.105	1.234	1.270	1.387	1.535
	Mean	1.382	1.375	1.321	1.383	1.395	1.447	1.542
Smg / Nmg ×10	Max.	1.076	1.018	1.040	0.865	0.791	1.036	0.902
	Min.	0.130	0.705	0.258	0.615	0.582	0.652	0.874
	Mean	0.476	0.852	0.650	0.736	0.684	0.844	0.888

Sulfer content in terms of S/N of liver protein of rats fed with DAB was far less than that of normally fed rats. The sulfer content grew higher, the more marked the macroscopical pathological change took place and that of non-neoplastic (cirrhotic) portion of the liver with hepatoma nodules was still larger than the latter and showed closely similar value to that of normal rat liver at this stage. When a hepatoma appeared S-N ratio reached the maximum.

On the contrary S/N in beef liver fed rats, one of the control groups, showed minimal value. Though we can not explain the reason how this remarkable fact had to do with the inhibiting action of beef liver feeding on liver cancer production, we may assume that the addition of certain amount of a readily absorbable and assimilable sulfer compound to the diet may have some influence on production of liver cancer. It may be recalled that there have been studies of many researchers who added on DAB feeding of rats sulfer containing amino acids to the diet.

On observation of nitrogen content of hepatic protein (Fig 2.) though there was no discrepancy between two control groups, we reached also to an assump-

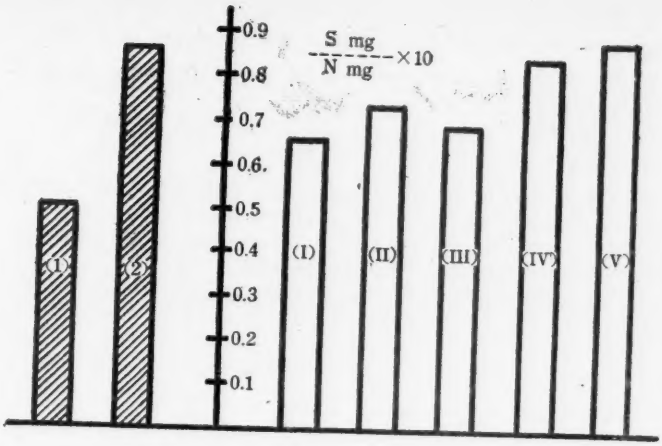

Fig. 1.

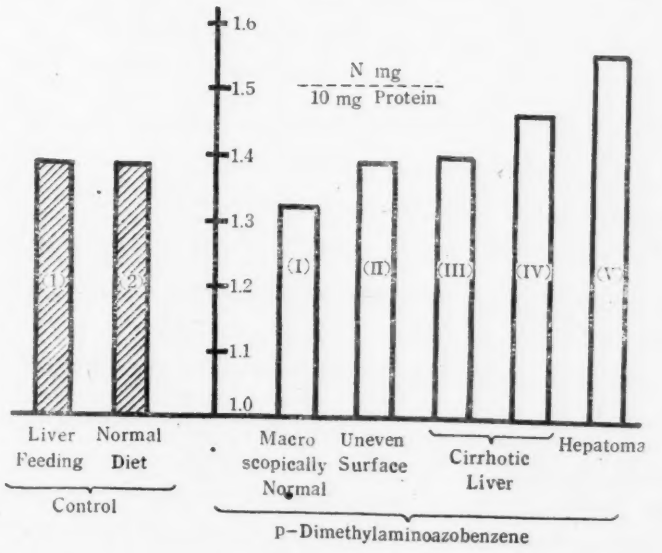

Fig. 2.

tion that it increased keeping steps with the extent of pathological change. There was seen fair coincidence with the result that Nakano [4] has obtained in the fraction of hepatic protein precipitated by trichloro-acetic acid.

The fact that a stepwise increase of nitrogen has become demonstrable is due to the above mentioned classification method based on pathological changes, instead of on feeding days with DAB.

It may be added here that a preliminary report on nitrogen determination in water extractable protein of entirely similar materials as used above has been published.[5] In that study nitrogen content grew lower the more the pathological change developed in hepatic tissue.

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要　旨

バターイエロー投與白鼠の肝蛋白の硫黄量に就て

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蛋白分子中の S-S 結合は分子の一定の形態を維持することに一役を担っていると考えられるので，実驗的肝癌生成過程における肝蛋白のS量の消長を知ることにより，正常組織の惡性化への蛋白の形態的変化を窮知し得るものかと本実驗を行った。

白鼠にバターイエローを投與し100日以上経過したものを殺し，肝を肉眼的に觀察してその病変度により5組に分類し，対照動物には普通食(白米)の正常白鼠の肝と牛肝末投與40日の白鼠の肝の組を選んだ。

新鮮組織を粥状となし除濕器中に減圧乾燥後摺って粉末とし，これを熱アルコールで処理したものにつき S を定量(リービヒードメニール法)した。また同一粉末の N を定量(ミクロキールダール法)し，S/N の比を得て各組ごとの平均値を檢討した。

バターイエロー投與により肝蛋白の S/N は普通食の正常白鼠の肝より顯著に小となるが病変が肉眼的に認められるに至って大となり，肝癌結節の明らかに存在する肝の非癌性部(硬変)では更に大となり，白米食白鼠の肝と数値は近似する。而して肝癌は最大値を示した。対照の一つである白米に牛肝末添加投與の白鼠肝は最小値を示した。この顯著な事実と牛肝末の肝癌抑制作用との関連性は現在説明できないが，食物中にもし易く吸収され同化される S 化合物の或量が添加されればアゾ色素投與による肝癌生成に何等か影響あるものと想像される。諸学者の行った含Sアミノ酸投與の研究が想起される。尚肝蛋白 N 量は病変の亢進に平衡して増加するという明らかな結果が得られた。

(文部省科学研究費による)

[GANN, Vol. 42; March, 1951]

A SIMPLE SQUASH TECHNIQUE APPLICABLE TO THE CHROMOSOMES OF MAMMALIAN TISSUE AND TUMOR CELLS

(With Plate V)

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Recently, particular attention has been given to the view that the cytological study takes important part in cancer research, for the elucidation of many fundamental problems which are now in confusion. Most of the past work in this field have been done on sectioned material subjected to the classical paraffin method, and by this method the tumors have generally been treated as difficult material for the cytological study. Indeed, our experience proved that the paraffin technique showed many difficulties in application for tough material such as tumors, with many shortcomings in the results. It is patent to the cytologist that the study of this field in cancer is fraught with so many technical difficulties that progress has been slow and hesitant. Our knowledge on cytology of cancer is, therefore, much less satisfactory than in other fields of cancer research. The application of the improved squash or smear technique will promise further progress of study in this field.

Recently, some investigators (Koller 1942, 1947; Papanicolaou 1942, 1943; Dalton 1947; Timonen & Therman 1950.) have adopted these techniques in their studies of human cancer, producing important results.

The present author, while working with the hope of finding the method useful for the cytological study in tissue cells, developed a simple squash technique which is capable of giving excellent results for studying the chromosomes in various tissues and tumors of white rats, and probably of other mammals.

The new technique here adviced consists of the combination of preliminary fixation with acetic alcohol and staining with aceto-orcein, -gentianviolet or -lackmoid, as described below.

For the present technique the following solutions are required:

(1) Acetic alcohol as a fixative consists of 1 part of glacial acetic acid and 3 parts of 95% ethyl alcohol.

(2) The staining solutions are prepared as follows:

Contribution No. 250 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Aceto-orcein solution: dissolve 1 g of orcein in 45 cc of hot glacial acetic acid and then add 55 cc of dist. water; leave till cold and filter.

Aceto-lackmoid solution: dissolve 1 g of lackmoid in 45 cc of hot glacial acetic acid and then add 55 cc of dist. water as above.

Aceto-gentianviolet solution: dissolve 0.75 g of gentianviolet in 45 cc of hot glacial acetic and then 55 cc of dist. water as above.

Technique

The method is successfully applicable to any tissue which is removed from young embryos, adult testes or tumors. The tissue is cut into small pieces of about 2-3 mm^3 in size immediately after removal and fixed with acetic alcohol for 20 minutes to one hour at room temperature. Treatment for too long a time of fixation makes the tissue too hard to be mashed out later. The suitable time can be learned by practice. When necessary, the material can be stored in 10% acetic acid after fixation, but a longer storage makes the material tough.

After fixation, the tissue is transferred to 10% acetic acid in which it is left for about 5 minutes. Then, it is immersed again in 50% acetic acid for 15 to 30 minutes. These treatments assist in the softening of the tissue as to be favorable for squashing.

The tissue, after rinsing with acetic acid as above, is next stained by applying solution of aceto-orcein, aceto-lackmoid or aceto-gentianviolet. In general, staining should be finished at maximum of 50 minutes. The time of staining varies depending on different types of material. The length of time allowed for staining is one of the critical points of the technique. Some examples are given below, but the suitable time should be learned by experience.

Stains / Material (rat)	Length of time desired in staining (minutes)		
	Aceto-orcein	Acetogentianviolet	Aceto-lackmoid
	min.	*min.*	*min.*
Liver	15-20	5-7	30-40
Bone marrow	10-15	10-15	20-25
Kidney	20-25	15-20	30-40
Heart	30-45	20-30	40-50
Lung	20	10	30
Spleen	15-20	5-7	25-30
Brain	10-15	5	20
Hepatoma	25-40	10-20	30-45

After staining, the material is transferred to a clean slide along with a drop

of stain, and dissected into thin strips of tissue. The cover slip is placed over the material, and pressure is applied until it has reached the desired thickness. A flat plate of material may be obtained by carefully applying an even pressure on the cover slip under several layers of blotting paper.

To seal the cover slip, an imploved sealing medium recommended by Makino (1938, 1950) is to be used. This medium consists of nearly equal parts of hard paraffin and Canada balsam, melted together by gentle heat. For applying to the cover slip a heated spatula is used.

The preparation is then ready for study. These temporary preparations, when completely sealed, will keep without further deterioration from two to four months, if they are kept in a cool dark place. In microscopical examination it is desirable to use a green filter.

Remarks

In general, by the application of this new technique, the chromosomes are preserved with a well-defined, sharp outline, and extreme clearness of appearance. The chromosomes are found arranged well apart from one another; there in no sign of irregular agglutination or clumping together of chromosomes even in the case of minute size, nor is there shown any other obscure configuration which would make accurate observation difficult. The chromosomes generally show a tendency of being slightly thickened, but this makes no obstacle at all for observation. Thus, the counting of chromosomes and the study of their morphology and behaviour can be made with accuracy and easiness.

There is, however, a little variation in the results of staining according to the dyes employed. As recommended by La Cour (1941), aceto-orcein is highly effective with these preparations and gives a sharp differentiation of the chromosomes from the cytoplasma. Very notable and interesting is the fact that gentianviolet can also be used with good success in mixture of acetic acid, in the formula as adviced above. This mixture, namely, aceto-gentianviolet, stains the chromosomes in intense colouration, but is somewhat less sharply selective for chromosomes than is the former. Furthermore, it is noteworthy that the substitution of aceto-gentianviolet for aceto-carmine likewise gives satisfactory results in the case of temporary smears with fresh material of many animals. As compared with the above two stains, aceto-lackmoid gives a little pale colouration in staining, and is less excellent in the results.

The technique here presented has several advantages, of which the most important is that in the material prepared with this method both cells and chromosomes are considerably prevented from shrinking that generally occurs in paraffin sections. Consequently, the chromosomes by this technique are surpri-

singly larger in size than are those prepared by the paraffin method. This evidence may be clearly understood by comparing Figures 1-6 drawn from the squash preparations with Figure 8 from the classical paraffin section, under the same scale. Simplicity and speed of procedure are also other advantages of this technique.

The results obtained by the application of this technique probably may be appreciated more precisely by referring to the accompanying figures (Figs. 1-7; Figs. 9-14) than by any descriptive explanation.

The work was done under the guidance of Professor Sajiro Makino to whom the author wishes to express his cordial thanks for helpful advice and for revising the manuscript.

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EXPLANATION OF PLATE V

Figs. 1-8; camera-lucida drawings of chromosomes of white rats. 1-7, from squash preparations stained by aceto-orcein, and 8 from paraffin section stained by iron-haematoxylin. ×2600. **1**, spermatogonium from adult testis. **2**, bone-marrow cell from young embryo. **3**, cell from heart (young embryo). **4**, cell of amnion. **5**, tumor cell from hepatoma showing regular number of chromosomes. **6**, the same containing semi-tetraploid chromosomes. **7**, the same showing tetrapolar divisions. **8**, cell of liver (young embryo), from paraffin section.

Figs. 9-14; photomicrographs of chromosomes of white rats, from squash preparations. **9**,spermatogonial cell. **10**, cell from brain. **11**, cell from kidney. **12**, cell from hepatoma. **13**, the same. **14**, polypolar divisions of hepatoma cell. 9-12; × 1800, 13; × 1200. 14; × 6000. All stained with aceto-gentianviolet. *Photo by Dr. S. Makino.*

抄　　錄

哺乳動物の体細胞ならびに腫瘍細胞の染色体研究に用いられる簡單なる“押しつぶし法”

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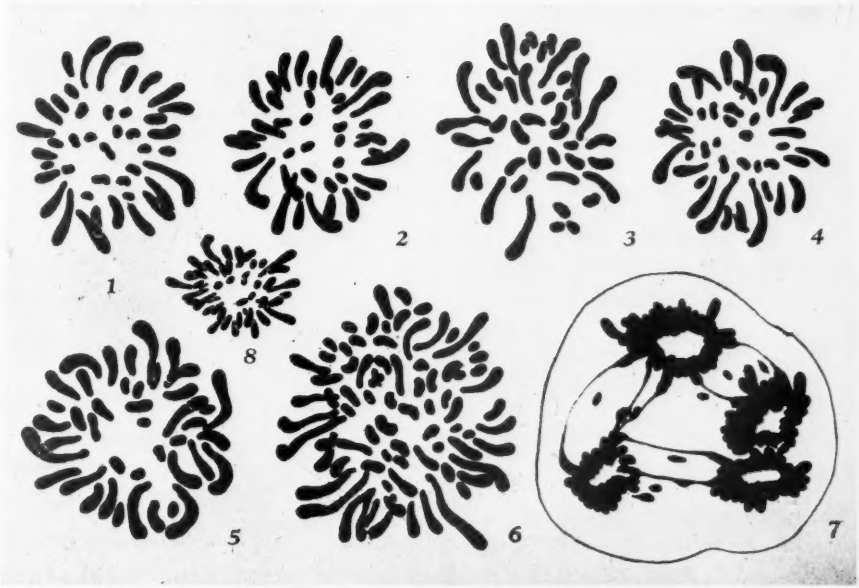
現在迄の癌の細胞学的研究は，主に固定材料を以てするパラフィン切片法に依って行われて來たが，この方法は実施上その操作が非常に煩雑であり，時間的にも不経済で，また体細胞ことに癌細胞のように非常に緻密な構造をもつ組織細胞の染色体固定には幾多の困難が存在し，固定液の選定と使用法に経験を必要としなければならない。この技術上の煩雑が癌の細胞学的研究の発展に非常な障害となっていることは，何人も等しく認めるところであろう。ここに塗抹法とか，押しつぶし法のような実施に時間のかからない簡單なテクニックの應用が考えられる所以である。

筆者は吉田肉腫にアセトカーミン法を應用してその染色体を観察しているが，この仕事に従事している間に，間質が液状である吉田肉腫の染色体が非常によく氷醋酸で固定されることにヒントを得て，他の組織においても固定後，間質を軟解することが出來れば，体細胞のような緻密な組織細胞にも押しつぶし法が適用出來るように思われた。そして主にラッテの胎兒の各種組織細胞とアゾ肝癌を用いて実驗した後，染色体を観察するのに簡便なこの方法を考案した。

固定液はアルコールと氷醋酸を 3 : 1 の割合に混合したものが最も良好である。固定後 10% 氷醋酸に 5 分，次に 50% 氷醋酸に 20～40 分処理して固定の爲硬化した組織を軟解する。そして，次の染色液の何れか一つによって染色する。(Aceto-orcein, Aceto-lackmoid, Aceto-gentianviolet)。染色後，組織片をスライドの上に取り，數片にきざんでからカバーグラスを上からかけて押しつぶし，余分の染色液を吸引の後カバーグラスの周辺を，バルサムパラフィンで封じて直に檢鏡出來る。

この方法は簡單な試藥を以て短い時間で仕上げることが出來，パラフィン法における細胞の收縮が最小限に止めることが出來るので，染色体は切片材料で見ることが出來ない程大きく観察が非常に樂である。染色体は幾分太目にはなるが，輪廓はシャープで観察に支障はない。尙，この実驗中に考案した Aceto-gentianviolet は，新鮮材料の塗抹標本にアセトカーミンの代用として良好なる結果を示すことを附記する。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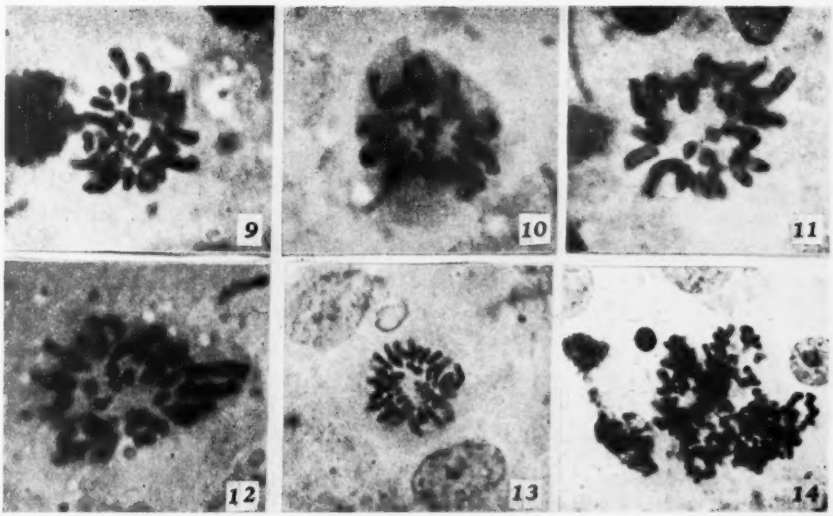
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[GANN, Vol. 42; March, 1951]

SOME OBSERVATIONS ON THE CHROMOSOMES IN THE YOSHIDA SARCOMA CELLS BASED ON THE HOMOPLASTIC AND HETEROPLASTIC TRANSPLANTATIONS (A PRELIMINARY REPORT) (With Plate VI)

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The Yoshida sarcoma has been well known as an ascites-tumor specific to the white rat (*Rattus norvegicus*), characterized by tumor cells which show a remarkable host-specificity in their malignant development (Yoshida, 1949).

Based on the analysis of daily frequencies of abnormal mitosis occurring in tumor cells together with their mitotic rate, which were observed throughout the whole life day of the host, the author expressed the view[2] that tumor cells which are undergoing regular division contribute primarily to the growth of the tumor, and further that these cells are highly characterized by their chromosomal peculiarity not only in the number but also in the morphological characters of the complement. Namely, they possess a chromosome number closely approximate to the normal one, but fluctuating between 39 and 42. Moreover, they are very remarkable by having a peculiar chromosome complex, characterized by the occurrence of a prominent V-element of large size together with many small V-shaped ones, more or less than 16 in number. On account of these characteristics the chromosomes of tumor cells show a marked differentiation from those of the host animal, *Rattus norvegicus*, since in the latter form have decidedly been established 42 chromosomes, all of which are of rod-type showing no V-element (Makino 1943, Tanaka 1951). Figures 2 and 3 show chromosomes of tumor cells developed in the ascites of the white rat, while Figure 1 indicates those of a bone marrow cell of the same animal. If now we compare the chromosome complex between the former and the latter, the dissimilarity is at once apparent; the morphological difference of chromosomes existing between them is particularly striking. The demarkation in the chromosome morphology between them is very distinct, and so far as the observations go, there has been demonstrated no transitional type of chromosomes between normal cells and tumor cells.

1) Contribution No. 252 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan. Aided by the Scientific Research Fund from tne Ministry of Education.

2) At the 22nd Annual Meeting of the Genetics Society of Japan at Tokyo, October 1950, the author delivered an address in this respect.

The present study was undertaken with the view to see the chromosomal condition in tumor cells which were transplanted into heterologous animals other than the specific host (*Rattus norvegicus*), since the results from this investigation may be of importance in connection with the view concerning the individuality of tumor cells expressed by the author. By means of intraperitoneal injection, the tumor ascites out of the white rat was transplanted into some heterologous animals, such as the house mouse (*Mus musculus*), the field mouse (*Apodemus geisha*), the black rat (*Rattus rattus*), the vole (*Clethrionomys bedfordiae*) and the guinea pig (*Cavia cobaya*). In every case of these heteroplastic transplantations, the introduced tumor cells could continue to live in the peritoneal cavity of these heterologous hosts for a certain duration and showed mitotic division in more or less degree. But they all disappeared later and the hosts remained alive. The tumor ascites was taken from these animals a few days after transplantation and smeared with acetocarmine.

The investigation carried out, based on these smear preparations, revealed that the chromosomes observed in tumor cells which were derived from these heterologous animals as given showed nothing visibly different from those found in tumor cells from the homologous host (*R. norvegicus*), both in their numerical relation and in the other morphological features. Examples are given in Figures 4 to 9. Referring to these figures, it is evident that every metaphase plate shows a chromosome number fluctuating between 39 and 42, and also they are very remarkable by the presence of a characteristic large V-element along with small V-shaped ones, nearly 16 in number. Comparison of Figures 2-3 with Figures 4-9 discloses a striking morphological similarity of chromosomes between tumor cells derived from the homologous host and those from the heterologous hosts. Furthermore, there is again a close similarity of the chromosomes between any two figures within the heteroplastic transplantation. The evidence is at once apparent by comparing the chromosomes in their serial alignments as seen in Figures 10 to 15; a striking uniformity is demonstrated with extreme clearness among all the cases from the heteroplastic transplantation as well as the homoplastic transplantation. The above evidences seem to show that tumor cells, which were introduced from the white rat into the heterologous hosts and proliferated there for a few days, are no other than those originated from the tumor of the white rat. At the same time, it is possible to say that tumor cells of the white rat persist in their characteristic individuality concerning chromosomes during through the course of multiplication, even if they are introduced into heterologous animals.

By way of conclusion, it can be said on the basis of the above facts that there is a definite strain of tumor cells which possess a peculiar chromosome

complex characteristic to this tumor and play an important role in the growth of the tumor by their healthy division, and further that the chromosomal individuality of these tumor cells is kept unaltered from their original ancestor, through successive transplantations from generation to generation. Various mitotic abnormalities are of common occurrence in this tumor too; they are, as it seems to the author, derivatives of these strain cells which were produced through abnormal mitosis caused by the alteration of the normal spindle mechanism, the structural change of chromosomes and other unknown causes.

Detailed accounts will be published elsewhere in near future.

The author's cordial obligation is due to his coworkers, Messrs. T. Yoshida, T. Tanaka, H. Nakahara and Miss K. Kano, for their kind assistance in various ways given during the course of the study.

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Explanation of Plate VI

All are camera-lucida drawings, × 2000. Fig. 1, chromosomes of bone-marrow cell. Figs. 2-3, chromosomes of Yoshida sarcoma, from homoplastic transplantation in white rat. Figs. 4 to 9, chromosomes of sarcoma cells, transplanted heteroplastically in *Rattus rattus* (Fig. 4), *Mus musculus* (Figs. 5-6), *Apodemus geisha* (Fig. 7), *Clethrionomys bedfordidae* (Fig. 8) and *Cavia cobaya* (Fig. 9). The chromosome numbers in each are shown as follows: **42** (Fig. 1), **41** (Fig. 9), **40** (Fig. 3), **38** (Fig. 4), **41** (Figs. 5-7), **42** (Figs. 8-9).

Serial alignments of chromosomes

Fig. 10, chromosomes of tumor cell from homoplastic transplantation in white rat. Figs. 11-15. chromosomes of tumor cells, transplanted in heterologous animals, such as *Rattus rattus* (Fig. 11), *Mus* (Fig. 12), *Apodemus* (Fig. 13), *Clethrionomys* (Fig. 14), and *Cavia* (Fig. 15).

要　旨

異種移植をした吉田肉腫における染色体研究

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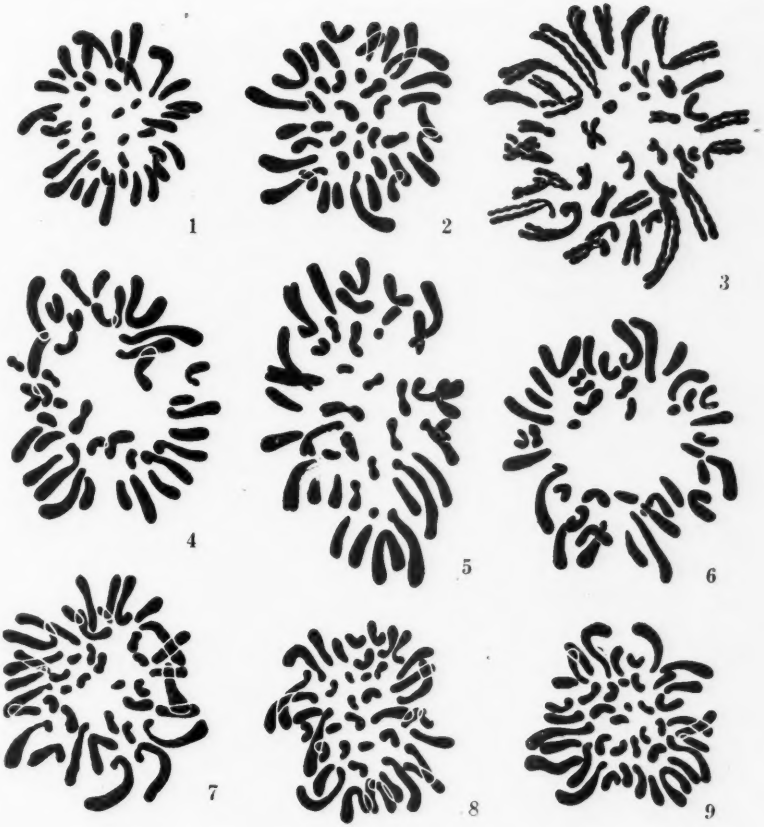
吉田肉腫の腫瘍細胞における異常分裂の出現頻度ならびに細胞分裂頻度の統計的調査の結果から著者は，吉田肉腫には40内外のほぼ一定した染色体数をもち，且特殊な核型（宿主のシロネズミのそれとは著しく異った）をもった一連の腫瘍細胞があって，これらが腫瘍の惡性増殖にあたって第一次的に重要な役割を演じているということを明らかにした（日本遺傳学会第22回大会発表，1950年10月）。吉田肉腫はシロネズミに特有な腫瘍であるが，シロネズミ以外の近緣動物に移植を行った場合における腫瘍細胞の染色体の狀態を知ることは，上にのべた知見を檢討する上に重要である。クマネズミ，ハツカネズミ，ヒメネズミ，エゾヤチネズミ及びテンヂクネズミ（モルモット）の腹腔に移植したシロネズミの吉田肉腫細胞は，これら異種動物の腹腔内で，いづれも或る期間生活し，少数ながら分裂した。これらの細胞において染色体をしらべると，シロネズミに累代移植を行った腫瘍細胞におけるものと，染色体数においても核型においても何等変る所をみない。第2-3図はウイスター系シロネズミ（*Rattus norvegicus*）に累代移植の腫瘍細胞における染色体の一例で，第4-9図は夫々クマネズミ（*R. rattus*，第4図），ハツカネズミ（*Mus musculus*，第5-6図），ヒメネズミ（*Apodemus geisha*，第7図），エゾヤチネズミ（*Clethrionomys bodfordiae*，第8図）及びテンヂクネズミ（*Cavia cobaya*，第9図）に移植された腫瘍細胞において觀察した染色体である。シロネズミ移植と異種移植の間においても，又異種移植同志の間においても，その間に染色体数に或は核型に何等の相違を発見し得ない。凡ての場合，一様な染色体構成が認められる。

これらの結果から，異種移植において異種の宿主の体内に或る期間増殖する腫瘍細胞は，もとのシロネズミの腫瘍細胞に由來したものであることは疑問の余地がない。尚又これらの事実はシロネズミにおいて累代移植に当って腫瘍の増殖に一次的な役割を果すものは，ほぼ一定した染色体数と特殊な核型をもった一連の腫瘍細胞であるという知見を裏書するものである。腫瘍増殖の期間に出現する，いろいろな種類の異常細胞は，これら一連の根幹細胞が，或る原因によつて変性して生じたものであることは別の実験によって明らかにされている所である。以上の詳細は他日公表する。　（文部省科学研究費による）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